AD NUMBER

ADB282177

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 2001. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, dtd 21 Feb 2003

AD_____

Award Number: DAMD17-98-1-8107

TITLE: Growth Inhibition of Breast Tumor Cells by Hypodense and Normodense Eosinophilic Cell Lines

PRINCIPAL INVESTIGATOR: Paulette M. Furbert-Harris, Ph.D.

CONTRACTING ORGANIZATION: Howard University
Washington, DC 20059

REPORT DATE: July 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8107
Organization: Howard University

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

7/1/02

_____ _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Final (15 Jun 98 - 14 Jun 01)

4. TITLE AND SUBTITLE
Growth Inhibition of Breast Tumor Cells by Hypodense and Normodense Eosinophilic Cell Lines

5. FUNDING NUMBERS
DAMD17-98-1-8107

6. AUTHOR(S)
Paulette M. Furbert-Harris, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Howard University
Washington, DC 20059

E-mail: pfurbert-harris@howard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We hypothesized that activated eosinophils (Eos) can inhibit the growth of breast tumor cells in vitro, and that this due in part to the action of released mediators. In this study we utilized Eos cell lines, previously developed from peripheral blood of allergic/asthmatic individuals, and newly developed sublines (FACS sorting of parent lines using antibodies to Eos markers). Using both monolayer culture and colocy forming assays, we have demonstrated that both hypodense (.22) and hyperdense (.24) cell lines and sublines markedly inhibited MCF-7 and MDA-MB-231 tumor cell growth. Inhibition of colony formation ranged from 3% to 100% at E:T ratios 10:1 to 1000.1. Using an integrated density value (IDV) measurement of the cell density in the monolayer cultures, inhibition ranged from 0% to 75% at E:T ratios 1:1 to 5:1 Conditioned supernatants, containing TNF-alpha and IL-4 inhibited colony formation by as much as 100%. These data clearly demonstrate the cytotoxic activity of Eos hypodense and hyperdense cell lines (comparable to activated peripheral blood eosinophils) which bind to tumor cells, release mediators that kill them. The establishment of Eos cell lines and sublines offer a rich resource for future studies on the characterization of the mediators (both protein and molecular characterization), the binding requirements and the overall biologic role of Eos as anti-cancer effectors.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
98

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

	Page Number
Cover	
SF 298	2
Introduction	4
Body	4
Key Research Accomplishments	9
Reportable Outcomes	9
Conclusions	10
References	11
Appendices	12

Introduction

Eosinophils are nonimmune inflammatory cells primarily found in allergic inflammatory reactions and parasitic infections (1). In addition to their granular proteins, [major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP)] which are toxic to surrounding tissues during inflammatory reactions, activated eosinophils produce an array of cytokines which are autologously regulating, and can thereby establishing a constitutive eosinophil presence at sites of inflammation (2-5). Many of these cytokines also have anti-cancer activities (2). Moreover, activated eosinophils also produce other granular proteins, perforin and granzymes and hence have the capacity to kill tumor targets similarly to cytotoxic T lymphocytes and natural killer cells (6). Eosinophils have been found in breast cancer infiltrates (7), however their active role in cancer remains ambiguous. In this investigation we have demonstrated that eosinophils, like NK cells may be non specific anti-cancer effector cells and that their mechanism of killing of tumor cells is two-tiered; l) by binding to and releasing toxic granular proteins like perforin and granzymes which cause the tumor cells to die by apoptosis and 2) by release of cytokines which inhibit tumor growth. In prior studies in this laboratory, hypo- and hyperdense eosinophils were collected from metrizamide density gradient fractions 22 and 24. These subpopulations of eosinophils were immortalized and characterized by flow cytometry using a panel of fluorescein-labelled antibodies to surface markers found on eosinophils (fig. 1.). We have demonstrated that these cell lines like fresh peripheral blood eosinophils inhibit MCF-7 and MDA-MB-231 breast tumor cell growth. We have also demonstrated the ability of conditioned media (cultured supernatants) and exogenous cytokines to inhibit tumor cell colony formation and we have quantified tumor cell destruction in monolayer cultures using an Integrated Density Values (IDV) method.
Moreover, we have evaluated eosinophil binding to and infiltration of MCF-7 multicellular tumor spheroids (MTS).

Body

Propagation of Cell Lines. To date all eight eosinophilic cell lines have been retrieved from storage at -160°C, cultured in RPMI medium supplemented with sodium pyruvate (1mM), non essential amino acids (1mM), penicillin/streptomycin (50 units/50ug/ml, respectively), gentamycin (50ug/ml) and 10% fetal bovine serum; and refrozen. At present we have data with four of the cell lines.

Growth Inhibition of MCF-7 and MDA-MB-231 tumor cells by Eosinophilic Cell Lines.
a) Monolayer. Tumor cells (both MCF-7 and MDA-MB-231) were seeded into T_{25} culture flasks at 3×10^5 cells/flask and incubated overnight, (16-24hr) at 37°C, 5% CO_2. Eosinophils were added at effector to target (E:T) ratios of 5:1 and 43:1 (for hypodense line SD.031.22) and 5:1 and 14:1 (for eosinophilic hyperdense line SD.031.24) and the flasks were incubated for 3 to 4 days. The effector cells were removed, the flasks washed with phosphate buffered saline then stained with hematoxylin and eosin. Data were captured by photomicrography.

Both cell lines SD.031.22 and SD.031.24 markedly inhibited both MCF-7 (fig. 2B, 2C, 2D and 2E, respectively); and MDA-MB-231 cells (fig. 3B, 3C, 3D and 3E, respectively). Results were similar when a third cell line, BJA.060.22 was tested against both MCF-7 (Fig. 4) and MDA-MB-231 (Fig. 5) tumor cells at E:T ratios of 1:1, (B) 20:1, (C) and 40:1, (D). In (fig. 6), peripheral blood eosinophil subpopulations hypodense (6B) and hyperdense (6C) confirm preliminary data of eosinophilic destruction of tumor cell growth, at E:T ratios as low as 2:1.

b) Colony Formation. MCF-7 tumor cells were seeded into the wells of a 6-well tissue culture plate at 500, 250, 100 and 50 cells/well to establish cloning efficiency and optimum concentration for the inhibition assay. The plates were incubated at 37C, 5% CO_2 for ten days, until the colonies were visible by the naked eye. The plates were harvested, washed with PBS then stained with hematoxylin and eosin, and counted manually. In (fig. 7), the percent cloning efficiency for the four cell concentrations (triplicate experiments) were 50, 52, and 56% (500 cells); 60, 64, and 72% (250 cells);140, 98, and 78% (100 cells) and 35, 45, and 80% (50 cells). From these data we originally selected both 250 and 100 cells, based on the percent efficiency, however from this point on we seed only 100 cells because of the facility in counting the colonies manually. (Fig. 8) shows colony inhibition of MCF-7 cells by peripheral blood hypodense and hyperdense (A and B) eosinophils respectively. At 100:1 E:T ratios, both eosinophil subpopulations inhibited MCF-7 tumor colony formation by 79% and 80%, respectively. Hypodense eosinophilic cell line BJA.060.22 (Fig. 9A) inhibited MCF-7 colony formation in a dose dependent manner at effector to target ratios of 10:1, 100:1, and 1000:1 (29%, 66% and 82%, respectively). The hyperdense cell line (BJA.060.24) inhibited colony growth by 33-44% at all ratios (fig. 9B). At 100:1 it required 10-fold more cells from the cell lines to inhibit colony growth by the same extent as did the fresh eosinophils. Against MDA-MB-231 tumor cells BJA.060.24 eosinophilic cell line (fig. 10B) inhibited colony formation dose dependently (3%, 43% and 70% at 10:1, 100:1, and 1000:1, respectively), while at 1000:1 BJA.060.22 (Fig. 10A) dramatically inhibited colony formation by 93%.

Growth Inhibition of MCF-7 and MDA-MB-231 tumor cells by Eosinophil Sublines.

Monolayer - MCF-7 and MDA-MB-231 tumor cells were seeded into the wells of a 6-well cluster plate at $1x10^5$ cells per well as previously described. The plates were incubated overnight at 37°C, 5% CO_2. Eosinophil sublines (generated by FACS sorting with eosinophil eotaxin-receptor antibody) at E:T ratios 1:1, 2:1 and 5:1. Cell line GRC.014.24.S1 was unable to inhibit the growth of MRC-5 fibroblasts at 1:1, 2:1 and 5:1 E:T ratios (fig. 11). On the otherhand, at 2:1 and 5:1, MCF-7 cells were inhibited. Cell line GRC.014.22S (fig. 12) completely abrogated MCF-7 growth at 1:1 and 2:1 E:T ratios (fig.13).

24 hr Supernatants From Peripheral Blood Eosinophils Inhibit MCF-7 Colony Formation.
Based on the above results we evaluated 24hr supernatants for inhibition of tumor colony formation (Fig. 14). Hypodense eosinophil fractions 22 inhibited MCF-7 colony formation by 30-50%. This inhibition was abrogated in all six of the samples when anti-IL-4 antibody was added to the tumor cells along with the supernatants.

Supernatants from hyperdense eosinophil fractions (24) inhibited MCF-7 colony growth by 26-48%, and anti-IL-4 antibody abrogated the activity in 4/6 of the samples. Conditioned supernatants (24, 48, and 72 hours) were further collected from 4 parent cell lines and 3 sublines and tested for inhibitory activity against MCF-7 and MDA tumor cells.

Collection of Cultured Supernatants From Eosinophil Cell Lines. Conditioned supernatants from eosinophil cell lines were then collected at various culture times in order to determine optimum release time for inhibitory agents into the cultured medium. Four parent lines and 3 sublines were cultured for 24, 48 and 72 hrs. The conditioned medium was collected at each culture time period and tested against MCF-7 and MDA tumor cells for inhibition of colony formation. Tumor cells were seeded into 6-well plates at 100 cells per well. The plates were incubated overnight at 37°C. Conditioned media supernatants from eosinophil cell line cultures (24, 48 and 72hrs) were added to the wells in total volume of 2mls. Serial 2-fold dilutions of the conditioned supernatants were made and each dilution was added in triplicate to the cells. Each plate contained triplicate control wells (10% RPMI medium). The plates were then incubated for 10 days at 37°C, 5% CO_2. The wells were washed carefully with phosphate buffered saline, then stained with hematoxylin and eosin. Manual counts were taken and percent inhibition determined. Concentrated undiluted supernatants completely inhibited colony formation (data not shown). With the 1:2 dilution of cultured supernatant from the hypodense cell line GRC.014.22 (fig.15), there was 73% inhibition of MCF-7 colony formation and 93% with both the 48hr and 72hr supernatants. At the 1:4 dilution there was 69% inhibition with the 24hr supernatant, 81% with the 48hr supernatant and 88% with the 72hr preparation. At the 1:8 dilution 30% inhibition occurred with the 24hr supernatant, 52% with the 48hr supernatant and 71% with the 72hr preparation. When MDA-MB-231 tumor cells were analyzed, the concentrated supernatants also inhibited these cells by 100% (data not shown). Although the inhibition of MDA colony formation was less than that of MCF-7, the pattern was very similar (fig.16). GRC.014.24 supernatants also showed patterns of inhibition similar to that of GRC.014.22 (figs. 17, 18) against both MCF-7 and MDA tumor cells. A similar pattern of activity though lower was observed with cell line SD.031.22 (fig.19) in that when the 24hr supernatant was tested on MCF-7 cells, at 1:2 dilution there was 60% inhibition, 55% at 1:4 and 25% at 1:8. The pattern of activity was the same for both the 48hr and 72hr cultured supernatants however the percent inhibition was higher, 74%, 70%, 38% (48hr); and 97%, 74% an 38% (72hr). Activity against MDA-MB-231 was very similar (fig.20). Cultured Supernatants from eosinophil cell line SD.031.24 inhibited MCF-7 (fig.21) tumor cells similarly to the previous cell lines GRC.014.22 (fig.15) and GRC.014.24 (fig.17) and SD.031.22 (fig.19). Inhibition by the 24hr supernatants was 92%, 60% and 54% for the 1:2, 1:4 and 1:8 dilutions, respectively. Inhibition by the 48hr supernatant was 98%, 88% and 75% for the same dilutions, respectively. The 72hr cultured supernatant showed the same pattern of inhibition. The inhibitory activity of SD.031.24 supernatants against MDA-MB-231 (fig.22) was less than that against MCF-7, similarly to the GRC.014.24 supernatants. Sublines of the GRC.014 cell lines were developed by sterile sorting with a Fluorescent Activated Cell Sorter using CCR3 (Eotaxin Receptor) and CD49d antibodies.

Supernatants (24hr, 48hr and 72hr) from FACS sorted eotaxin receptor positive GRC.014.22 cells (referred to as GRC.014.22.S) (Fig. 23) inhibited MCF-7 colony formation by 100% , 88% and 71% (1:2, 1:4 and 1:8, respectively), 100%, 91% and 64% (48hr); 100%, 84% and 77% (72hr). At 1:4 and 1:8 dilutions (fig.24) MDA-MB-231 cells were inhibited by ≥50%. Against MCF-7, the GRC.014.24S eotaxin receptor positive eosinophil supernatants markedly inhibited colony formation 90%, 92% and 96% from 1:2 dilutions of 24hr, 48hr and 72hr cultured supernatants (fig. 25). At 1:4 dilutions, inhibition was 50%, 75% and 85% for 24, 48 and 72hr cultures, respectively and at the 1:8 dilution percent inhibition was 26%, 54% and 63% for 24, 48 and 72hr cultured preparations, respectively. These supernatants also inhibited MDA-MB-231 cells (fig. 26). The last cell line tested was established from the GRC.014.24S subline (which is $CCR3^+$), using the FACS Sorter and second eosinophil marker, anti-CD49d. Cultured supernatants from these cell line also inhibited MCF-7 (fig. 27) and MDA-MB-231 (fig. 28) in a dose-dependent manner.

Cytokine Presence in Eosinophil Cultured Supernatants.
24 hr supernatants from peripheral blood eosinophil hypodense and hyperdense fractions (22 and 24, respectively), were evaluated by enzyme-linked immunoassay (ELISA) analysis, using commercial kits. Interleukin-4 (IL-4) and TNFα were present in varying levels in all six individuals tested (Table 1). IL-4 concentrations ranged from 0 to >1000 pg/ml, while TNFα concentrations, which were far less than IL-4, ranged from 10 - 224 pg/ml. GM-CSF was only found in donor 6 at 450 pg/ml, and IL-5 was absent in all 6 samples. Moreover, when 24hr, 48hr and 72hr cultured supernatants from eosinophil cell lines (parent and sublines) were evaluated, TNFα was found in all samples at >40 pg/ml concentration (data not shown).

Effect of Exogenous Cytokines on MCF-7 Colony Formation
IL-3 (fig. 29) at 10ng/ml inhibited MCF-7 colony formation by 15%; at 50ng/ml by 30%, 0% at 100ng/ml and 26% at 200ng/ml. IL-4 inhibited colony formation by 10% at 10ng/ml, 25% at 50ng/ml and 14 at 100ng/ml. IL-5 at 10ng/ml failed to inhibit colony formation at 50 and 100ng/ml exerted 20% inhibition. At 10ng/ml IL-3 inhibited MDA-MB-231 (fig. 30) colony formation by 10%, but had little to no effect any of the higher concentrations. At 10ng/ml, IL-4 inhibited colony formation by 17%, 28% at 50ng/ml, 20% at 100ng/ml and 16% at 200ng/ml. At 100ng/ml IL-5 inhibited tumor growth by 36%. TNFα on the otherhand, dramatically inhibited colony inhibition of MCF-7 cells (fig. 31).

Integrated Density Value Measurement of the monolayer assay- In order to quantitate the inhibitory activity observed in the monolayer assays, we utilized a Chemi-Imager 4000 (alpha Innotech Corp) to measure the density of the cells in the wells. The well area was selected and saved as the spot overlay. This spot overlay was used on each well of a plate in order to standardize the area of all wells. As wells were selected, their Integrated Density Value (IDV) was automatically calculated. The IDV is the sum of all pixel values after background correction.

Auto background correction uses the Alpha Ease program which determines the average of the 10 lowest pixel values in each individual well and assigns that value as background. However, if no background is selected the background value is reported as zero. Because the average (AVG) is

equal to IDV divided the overlay value (which is constant), we used AVG as a comparative figure. We then compared the IDV of the control with that of the test samples. The percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{average IDV (Control) - average IDV (test)}}{\text{average IDV (control)}} \times 100$$

MRC-5 fibroblasts and tumor cells (MCF-7 and MDA-MB-231) were seeded into the wells of either a 6-well plate (at 5 x 10^5 cells per well) or a 12-well plate (at 1.5 x 10^5 cells per well) as previously described. The plates were incubated overnight (18-24hr) at 37°C, 5% CO_2 at 95% humidified atmospheric conditions before treatment: Eosinophils were added at various effector to target (E:T) ratios and the plates were incubated for an additional 72 hrs. The effector cells were then removed, the monolayers washed 3x with PBS and the wells stained with hematoxylin and eosin. At E:T ratio of 1:1, subline GRC.014.24S did not inhibit the growth of MRC-5 fibroblasts (fig. 32). As the E:T ratio increased the IDV increased giving a negative value of inhibition. Inhibition of MCF-7 and MDA-MB.231 tumor cells by sorted sublines, GRC.014.22S, GRC.014.24S, and GRC.014.24S/CD15 was obtained as the E:T ratios increased (fig.33, and 34). While this was comparable to that observed with fresh peripheral blood eosinophils against MDA (fig.36), it was not seen when MCF-7 tumor cells were tested (fig.35). In fact, as the E:T ratio increased, the percent inhibition decreased. At the higher E:T ratios we observed clusters of eosinophils binding to MCF-7 cells. Killing thus seemed to have been masked by large numbers of these clusters resulting in high IDV's (fig. 35). We also measured the activity of TNF-α and cultured supernatants on MCF-7 and MDA tumor monolayers, using the IDV method. TNF-α at a concentration greater than 0.25mg inhibited MCF-7 cell tumor cell growth (fig. 37). This was blocked by anti-TNF-α at 200 ng/ml. Comparable inhibition was produced by 48hrs conditioned supernatants from cell lines (fig. 38 and 39). However, this was only partially blocked by anti-TNF-α.

Infiltration of MCF-7 Multicellular Tumor Spheroids (MTS) by Peripheral Blood Hypodense and Hyperdense Eosinophil Fractions and by Eosinophil Cell Line.

a. MTS Production. MCF-7 multicellular tumor spheroids were developed by slightly modifying the method of Yuhas et al (7). Briefly, subconfluent monolayer cultures which were maintained (at 100% relative humidity, 95% air, 5% CO_2 at 37°C) in 10% RPMI complete medium . After trypsinization and cell count, the cells were dispensed into T_{25} non-vented flasks (1×10^6 cells/flask) and rocked at 30 rpm at 37°C for 24- 48hr, after which time the spheroids were transferred to 100 mm petri dishes containing an overlayer of 0.3% noble agar in 10% RPMI medium. The dishes were then incubated at 37°C in a 5% CO_2 atmosphere for 7-14 days with regular feeding to study the growth characteristics of the MTS. (Fig. 40) shows photomicrographs of MTS in culture for 7 days. They range in size from 100 mm to 500 mm. Characteristic necrotic cores can be seen in A and C

b. Eosinophil: MTS Co-culture Assay. As early as 48hrs, thirty MTS were transferred to a fresh petri dish with agar overlay. Eosinophils were added at ratios of 10:1 and 100:1 eosinophil: MTS. The dishes were incubated for 7 days, then observed microscopically for eosinophil attachment.

Photomicrographs in (fig. 41) show fresh eosinophils, hypodense (A,C and E) and hyperdense fractions (B, D and F) bound to spheroids, while in (fig. 42) eosinophil cell line BJA.060.22 is seen bound to the tumor spheroid. MTS were also fixed in 4% glutaraldehyde and processed with en bloc uramyl acetate for electron microscopic analyses.. Transmission EM (Fig. 43) analysis revealed eosinophils inside the MTS, both just inside the surface area and in the core.

Key Research Accomplishments

- Retrieval of all 8 eosinophillic cell lines
- Demonstration of functional cytotoxic/cytostatic activity with 4 of the lines
- Establishment of Multicellular Tumor Spheroids (MTS)
- Infiltration of MTS by eosinophils
- Promotion to Associate Professor
- Development of 3 eosinophil sublines from 2 previously established parental lines based on FAC Sorting, using eosinophil markers (at this time only 2 sublines survived)
- Established inhibition of tumor growth by all 4 parent lines as well as 3 sublines

Reportable Outcomes

- Hypodense and hyperdense eosinophils infiltrate MCF-7 breast multicellular tumor spheroids. Furbert-Harris PM, Harris D, Vaughn T, Parish-Gause D, D, Dunston GM, Abdelnaby A, Laniyan I, and Oredipe O. Proc Am Assoc Cancer Res. 40:455, 1999.
- Furbert-Harris PM, Anderson D, Parish-Gause D, Vaughn T, Brown R, Laniyan I, Dunston GM, Abdelnaby A, and Oredipe O. Eosinophilic destruction of breast tumor cells in vitro is mediated by Interleukin-4. FASEB 13(4):A612, 1999.
- Activated eosinophil subpopulations kill breast tumor cells in vitro. Furbert-Harris PM, Howland C, Hunter KA, Laniyan I, Vaughn T, Dunston GM, Parish-Gause D, Harris D, Abdelnaby A, Anderson D, Brown R, Awich J, Sumner s and Oredipe O. Era of Hope Department of Defense Breast Cancer Research Program, Proceedings Vol. II p. 674.
- Stimulation by swainsonine of myocardial sulfhydryl levels in high-dose doxorubicin chemotherapy. OA Oredipe, I Laniyan, WM Griffin, D Parish-Gause, T Vaughn, WR Green and PM Furbert-Harris. FASEB l4(8) 1155.
- EBV-Transformed Hypodense and Hyperdense Eosinophil Cell Lines Kill MCF-7 and MDA-MB-231 Breast Tumor Cells In Vitro. Furbert-Harris PM, Laniyan I, Vaughn T, Parish-Gause D, Dunston GM, Harris D, Abdelnaby A, Hunter KA, Howland C, Brown R, Awich J, and Oredipe O. (Manuscript In Preparation)
- Hypodense and Hyperdense Eosinophils Infiltrate MCF-7 Multicellular Spheroids (MTS). Furbert-Harris PM, Laniyan I, Harris D, Vaughn T, Parish-Gause D, Dunston GM, Abdelnaby A, Hunter KA, Howland C, Brown R, Awich J, and Oredipe O. (Manuscript In Preparation)
- $CCR3^+$, $CD49^+$, and $CD15^+$ EBV Transformed Sublines Inhibit Breast Tumor Cells In Vitro. Furbert-Harris PM, Laniyan I, Vaughn T, Parish-Gause D, Dunston GM, Abdelnaby A, Hunter KA, Howland C, Brown R, Awich J, and Oredipe O. (Manuscript In Preparation)

Discussion/Conclusion

We hypothesized that activated esoinophils from peripheral blood and eosinophil cell lines inhibit breast cancer cell growth by releasing inflammatory substances that slow down the growth of the cells or are toxic to the cells, thereby causing their death.

In this phase we have analyzed seven eosinophil cell lines, parent and sublines which we have developed by EBV-immortalization of metrizamide density gradient fractions of hypo-(M22) and hyperdense (M24) eosinophils. These cells were obtained from the peripheral blood of individuals (prior study) with mild to moderate eosinophilia. We have collected cultured supernatants from these cell lines, (which have been determined to inhibit the growth of breast tumor cells in vitro), and analyzed them for their inhibitory action on MCF-7 and MDA-MB-231 tumor cells. Cultured supernatants (24hr, 48hr and 72hr) from all 7 cell lines dramatically inhibited colony formation of both MCF-7 and MDA-MB-231 tumor cells. Undiluted, preparations of the cultured supernatants completely abrogated the colony growth. Serial two-fold dilutions, 1:2, 1:4 and 1:8 inhibited tumor colony formation in a dose-dependent manner.

Supernatant preparations from 24hr, 48hr and 72hr cultures demonstrated inhibition dose-dependently. All supernatants were examined for TNFα and all contained >40 pg/ml. Exogenous cytokines which are known to regulate eosinophil differentiation and activation (I L-3, IL-5, GM-CSF) and IL-4 and TNFα, all of which can be produced by activated eosinophils, were examined for tumor cell growth inhibitory activity. There was marginal growth inhibition with all except TNFα. IL-3 at 50 ng/ml inhibited MCF-7 colony formation by 30% while IL-5 at 100 ng/ml inhibited MDA-MB-231 colony formation by 36%. The data were variable with regards to dose with IL-3, IL-4, IL-5 and GM-CSF. Overall, inhibition ranged from 0-35%. TNFα however, was markedly potent in its inhibitory activity.

Activated fresh peripheral blood eosinophils bound to MCF-7 cell line but not to MDA-MB-231. At high E:T ratio, we observed the same binding in cell lines when activated in the presence of IL-5 (5ng/ml). Activation of eosinophils resulted in up regulation of adhesion molecules (ICAM, VCAM). This facilitates binding of eosinophils to MCF-7 cells resulting in large numbers of clusters masking cell destruction when measuring the IDV. This clustering is only observed when using E:T ratios greater or equal to 5:1. The IDV measurement of monolayer assays is somewhat limited in its efficiency in that some times it overestimates wells with clusters even if destruction of the entire surface cam be observed.

These data confirm our hypothesis that mediators (cytokines, granular proteins) released by eosinophil cell lines inhibit tumor cell growth. Further analysis of eosinophil supernatants will be performed in order to better characterize the mediator participants active in eosinophil anti-cancer cytotoxic activity.

We have used three methods of evaluation to determine these inhibitory effects on breast tumor cells by eosinophils, (colony formation, photodocumentation and Integrated Density Value, with colony formation being the most sensitive. The establishment of eosinophil cell lines (that are not tumorous) offer the opportunity to study eosinophil biology, functional activity, discriminate between various toxic/cytostatic proteins, genetics of killer activity and other biological activities.

References

1. Gaga M, Frew AJ, Veronica AV and Kay AB. 1991.
2. Molet S, Gosset P, Vanhee D, Tillie-Leblond I, Wallaert B, Capron Ma and Tonnel AB. Modulation of cell adhesion molecules on human endothelial cells by eosinophil-derived mediators. Jl Leuk Biol 63:35l-58, 1998.
3. Ibiri NI, Siegel JP, Varricchio Fa and Puri RK. 1994. Expressions of high affinity IL-4 receptors on human melanoma, ovarian and breast carcinoma cells. Clin Exp Immunol 95(l):148.
4. Tepper RI, Coffman RL and Leder P. 1992. An eosinophil-dependent mechanism for the anti-tumor effect of interleukin-4 Science 257:548.
5. Nakajima H, Gleich GJ and Kita H. 1996. Constitutive production of IL-4 and IL-10 and production of IL-8 by normal peripheral blood eosinophils. J. Immunol 156(12):4859.
6. Elovic AE, Ohyama H, Sauty A, McBride J, Tsuju T, Nagari M, Weller Pf, Wong DT. 1998. IL-4 dependent regulations of TGF-alpha and TGF-beta 1 expression in human eosinophils. J. Immunol 160(12):6121.
7. Kuby J. Immunology (4^{th} ed) RA Goldsby, Thomas J Kinat. Barbara A Osborne (eds). WH Freeman & CO., 2000.
8. Samoszuk MK, Nguyen V, Gluzman I, Pham JH. 1996. Occult deposition of eosinophil peroxidase in a subset of human breast carcinoma. Am J Pathol 148(3):701-6.
9. Yuhas JM, Li AP, Martinez O, Ledman AJ. 1997. A simplified method for production and growth of multicellular tumor spheroids. Cancer Res 37:3639.

Personnel:

Paulette Furbert-Harris, Ph.D. - Principal Investigator

Ibrahim Laniyan (Salary)

Theresa Vaughn (Wage)

Debra Parish-Gause (Wage)

Appendices

Figure 1. Cytospin Preparation of Eosinophil Cell Line Stained with H&E

Figure 1. Legend

Figure 2. Inhibition of MCF-7 Tumor Cell Growth by Eosinophil Cell Line

Figure 2. Legend

Figure 3. Inhibition of MDA-MB-231 Tumor Cell Growth by Eosinophil Cell Line

Figure 3. Legend

Figure 4. Inhibition of MCF-7 Tumor Cell Growth by Eosinophil Cell Line

Figure 4. Legend

Figure 5. Inhibition of MDA-MB-231 Tumor Cell Growth by Eosinophil Cell Line

Figure 5. Legend

Figure 6. Inhibition of MCF-7 Tumor Cell Growth by Peripheral Blood Eosinophils

Figure 6. Legend

Figure 7. Plating Efficiency of MCF-7 Tumor Cells

Figure 7. Legend

Figure 8. Growth Inhibition of MCF-7 Tumor Cell by Peripheral Blood Hypodense and Hyperdense Eosinophils

Figure 8. Legend

Figure 9. Growth Inhibition of MCF-7 Colony Formation by Eosinophil Cell Lines

Figure 9. Legend

Figure 10. Growth Inhibition of MDA-MB-231 Tumor Cell Colony Formation by Eosinophil Cell Lines

Figure 10. Legend

Figure 11. Eosinophil Cytotoxic Activity on MRC-5 Fibroblast Cell Growth

Figure 11. Legend

Figure 12. Eosinophil Cytotoxic Activity on MCF-7 Cell Growth

Figure 12. Legend

Figure 13. Eosinophil Cytotoxic Activity on MDA-MB-231 Cell Growth

Figure 13. Legend

Figure 14. Inhibition of Colony Formation by 24hr Eosinophil Supernatants

Figure 14. Legend

Figure 15. GRC.014.22 Eosinophil Supernatants Inhibit MCF-7 Colony Formation In Vitro

Figure 15. Legend

Figure 16. GRC.014.22 Eosinophil Supernatants Inhibit MDA-MB-231 Colony Formation In Vitro

Figure 16. Legend

Figure 17. The Effect of GRC.014.24 Eosinophil Cultured Supernatants on MCF-7 Breast Tumor Cells

Figure 18. The Effect of GRC.014.24 Eosinophil Cultured Supernatants on MDA-MB-231 Colony Formation

Figure 17,18. Legend

Figure 19. Effect of SD.031.22 Eosinophil Cultured Supernatants on MCF-7 Colony Formation

Figure 20. Effect of SD.031.22 Eosinophil Cultured Supernatants on MDA-MB-231 Colony Formation

Figure 19,20. Legend

Figure 21. Effect of SD.031.24 Eosinophil Cultured Supernatants on MCF-7 Colony Formation

Figure 22. Effect of SD.031.24 Eosinophil Cultured Supernatants on MDA-MB-231 Colony Formation

Figure 21,22. Legend

Figure 23. Inhibition of MCF-7 Colony Formation by GRC.014.22S Supernatants

Figure 24. Inhibition of MDA-MB-231 Colony Formation by GRC.014.22S Supernatants

Figure 23,24. Legend

Figure 25. Cultured Supernatants from an Eotaxin Positive (CCR3+) Eosinophil Cell Line (GRC.014.24S) Inhibit MCF-7 Colony Formation

Figure 26. Cultured Supernatants from an Eotaxin Positive (CCR3+) Eosinophil Cell Line (GRC.014.24S) Inhibit MDA-MB-231 Colony Formation

Figure 25,26. Legend

Figure 27. Cultured Supernatants from CCR3+, CD49+ Eosinophil Cell Line Inhibit MCF-7 Colony Formation

Figure 27. Legend

Figure 28. Cultured Supernatants from a CCR3+, CD49+ Eosinophil Cell Line Inhibit MDA-MB-231 Colony Formation

Figure 28. Legend

Table 1. Cytokine Concentrations in 24hr Eosinophil Culture Supernatants (pg/ml)

Table l. Legend

Figure 29. The Effect of Cytokine on MCF-7 Colony Formation

Figure 29. Legend

Figure 30. The Effect of Cytokines on MDA-MB-231 Colony Formation

Figure 30. Legend

Figure 31. Effect of TNF-α on MCF-7 Colony Formation

Figure 31. Legend

Figure 32. Inhibitory Effect of Eosinophil Cell Line (GRC.014.242) on MRC-5 Fibroblast Cell Growth

Figure 32. Legend

Figure 33. Inhibitory Effect of Eosinophil Sublines on MCF-7 Cell Growth

Figure 33. Legend

Figure 34. Inhibitory Effect of Eosinophil Sublines on MDA-MB-231 Cell Growth

Figure 34. Legend

Figure 35. The Effect of Peripheral Blood Eosinophils on MCF-7 Cell Growth

Figure 35. Legend

Figure 36. The Effect of Peripheral Blood Eosinophils on MDA-MB-231 Cell Growth

Figure 36. Legend

Figure 37. The Effects of TNF-α and anti-TNF-α on MCF-7 Cell Growth

Figure 37. Legend

Figure 38. TNF-α is an Inhibitory Component of Eosinophil Cell Line Supernatants

Figure 38. Legend

Figure 39. TNF-α is an Inhibitory Component of Eosinophil Cell Line Supernatants

Figure 39. Legend

Figure 40. MCF-7 Multicellular Tumor Spheroids

Figure 40. Legend

Figure 41. Peripheral Blood Hypodense and Hyperdense Eosinophils Bind to MCF-7 Multicellular Tumor Spheroids

Figure 41. Legend

Figure 42. Hypodense Eosinophillic Cell Line Binds to MCF-7 Multicellular Tumor Spheroids

Figure 42. Legend

Figure 43. Eosinophil Infiltration of MCF-7 Multicellular Tumor Spheroid

Figure 43. Legend

Fig.1 Cytospin preparation of Eosinophil Cell Line stained with H&E

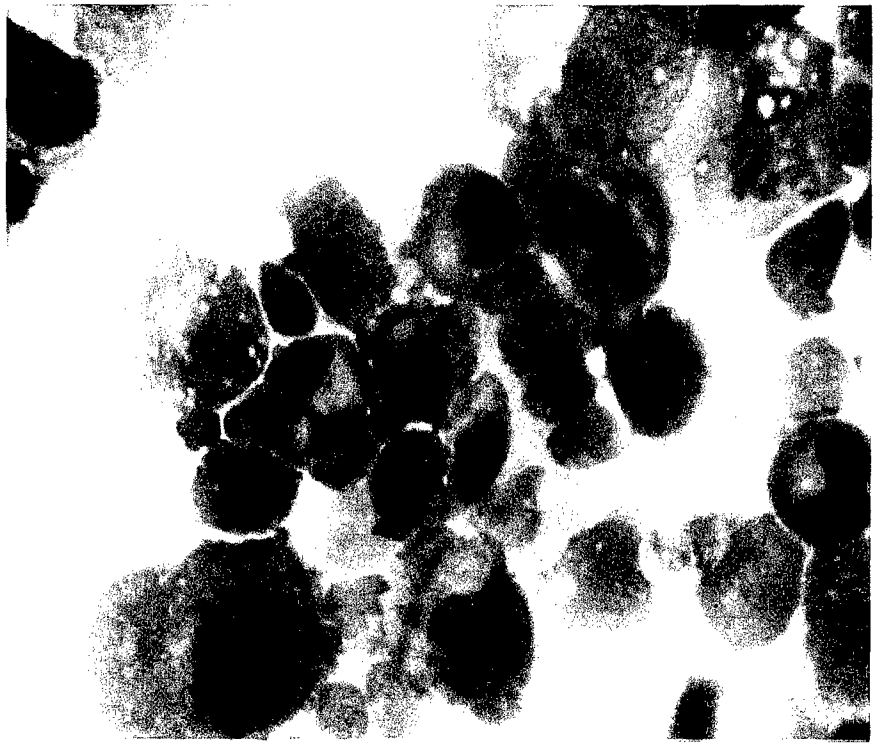


Fig 1. Cytospin preparation of eosinophil cell line stained with H & E

Fig. 2. Inhibition of MCF-7 Tumor Cell Growth by Eosinophil Cell Lines

Fig. 2. MCF-7 tumor cells were seeded into T_{25} flasks at $3x10^5$ cell/flask and allowed to grow to confluence (4-6 days) in media alone (A) or on the presence of hypodense eosinophillic cell line SD.031.22 at E:T ratios 5:1 and 43:1 (B and C) and hyperdense (D and E) cell line SD.031.24 at E:T ratios 5:1 and l4:l.

Fig. 3. Inhibition of MDA-MB-231 Tumor Cell Growth by Eosinophil Cell Line

Fig. 3. MDA-MB-231 tumor cells were seeded similarly to MCF-7 in media (A) and with SD.031.22 (B) and SD.031.24 (C) at similar E:T ratios 5:l, 43:l and 5:l, l4:l respectively..

Fig. 4. Inhibition of MCF-7 Tumor Cell Growth by Eosinophil Cell Lines

Fig. 4. Hypodense eosinophil line BJA.060.22 was co-cultured with MCF-7 tumor cells similarly to SD.031.22, however the ratios were l:l (B), l:20 (C) and l:40 (D) control tumor cells were cultured in media alone (A).

Fig. 5. Inhibition of MDA-MB-231Tumor Cell Growth by Eosinophil Cell Lines

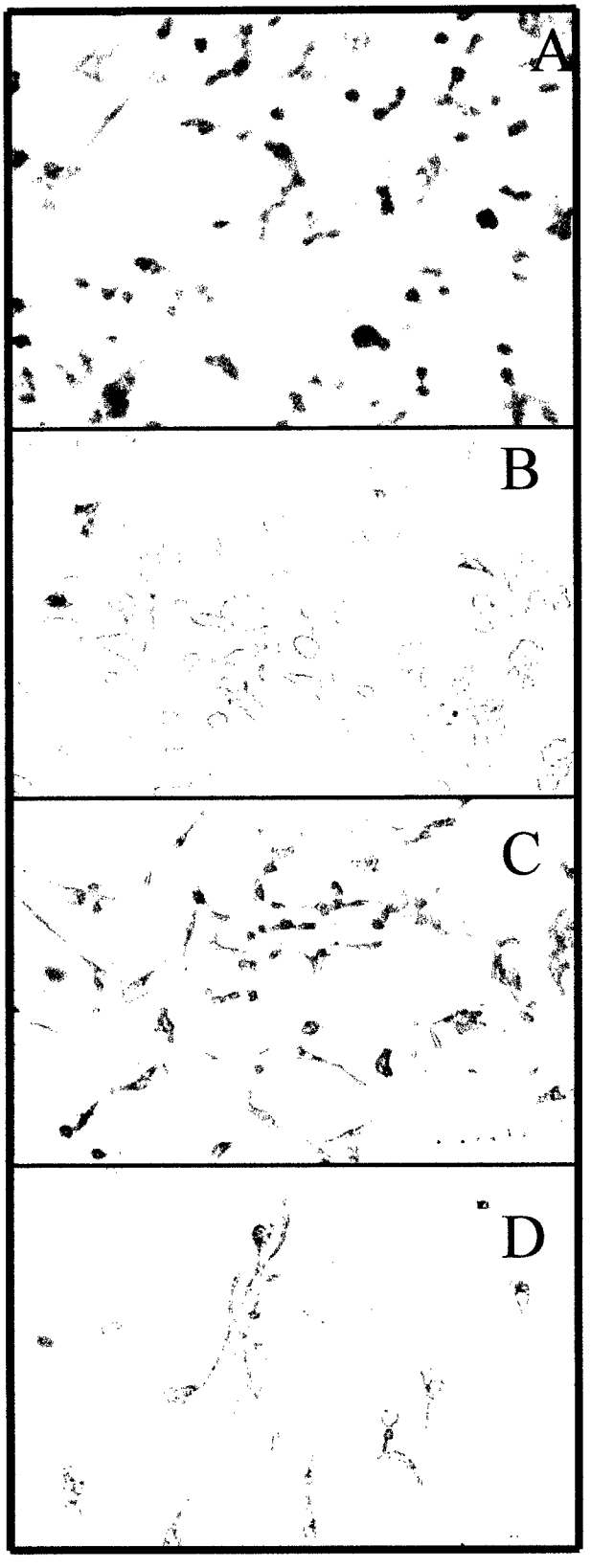


Fig. 5. Hyperdense BJA.060.24 was co-cultured with MDA-MB-231 tumor cells similarly to MCF-7 cells at E:T ratios l:l (B), 20:l (C) and 40:l (D). Control tumor cells were cultured in media alone (A).

Fig. 6. Inhibition of MCF-7 Tumor Cell Growth by Peripheral Blood Eosinophils

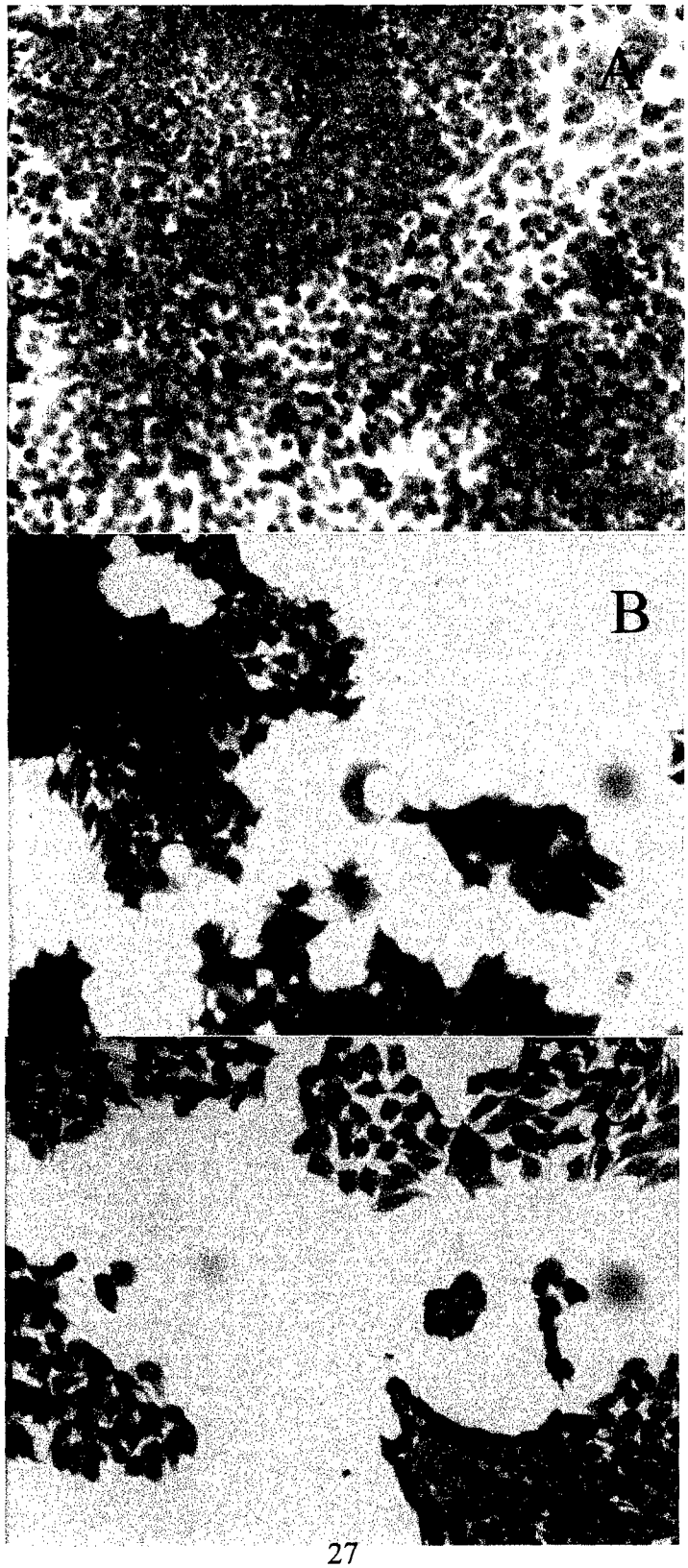


Fig. 6. Peripheral blood eosinophils, both hypodense (5B) and hyperdense (5C) show marked inhibition of cell growth when compared to that of the media control (5A) at 2:1 E:T ratios.

Fig. 7.

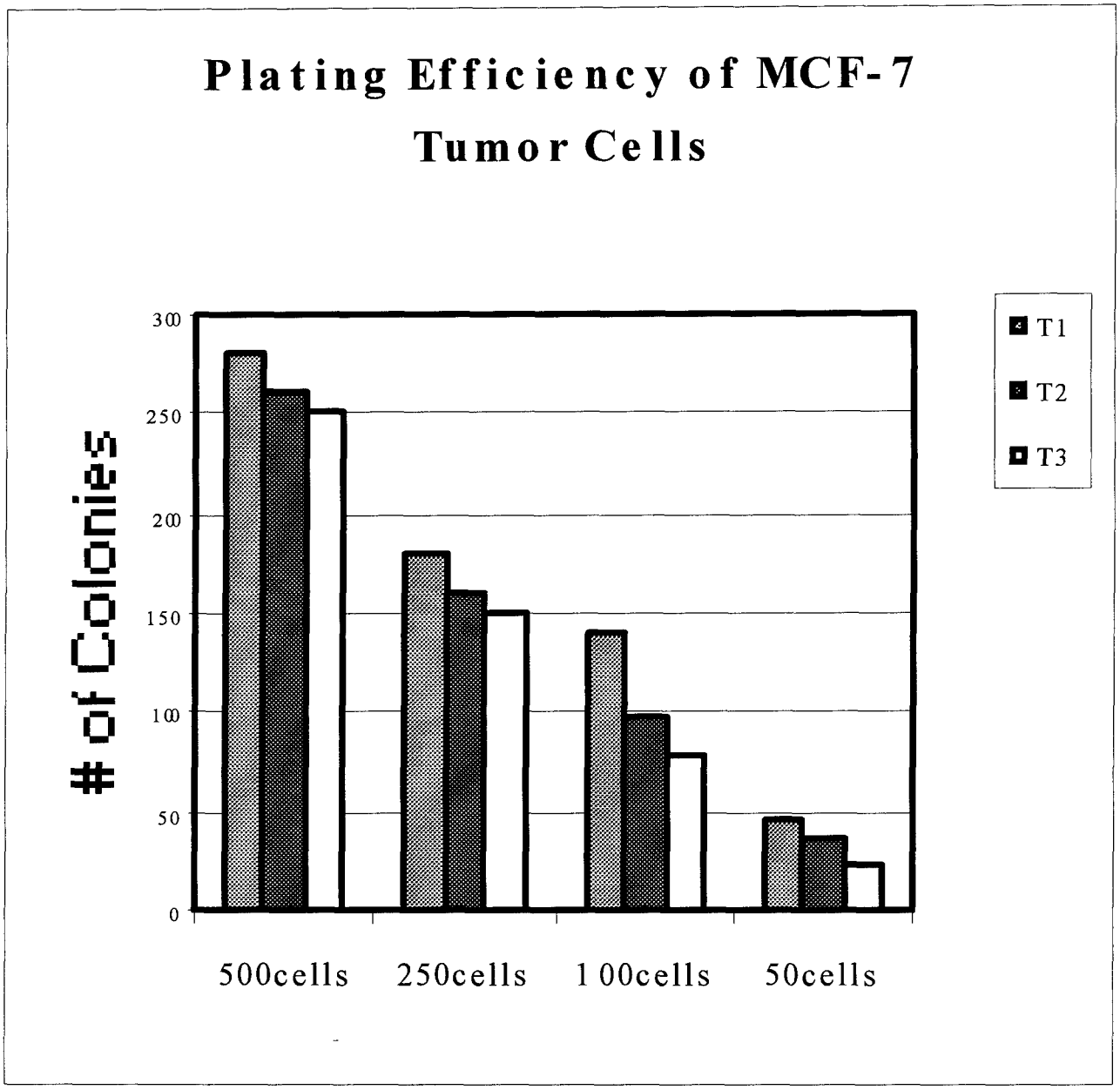

Plating Efficiency of MCF-7 Tumor Cells
# of Colonies
300
250
200
150
100
50
0
500cells
250cells
100cells
50cells
T1
T2
T3

Fig. 7. MCF-7 cells were seeded into 6-well plates at 500, 250, 100 and 50 cells per well. The plates were then incubated for 10 days at 37°C, 5% CO_2. Plates were washed 3X with PBS, then stained with hematoxylin and eosin.

Fig. 8 Growth Inhibition of MCF-7 Tumor Cells by Peripheral Blood Hypodense and Hyperdense Eosionphils

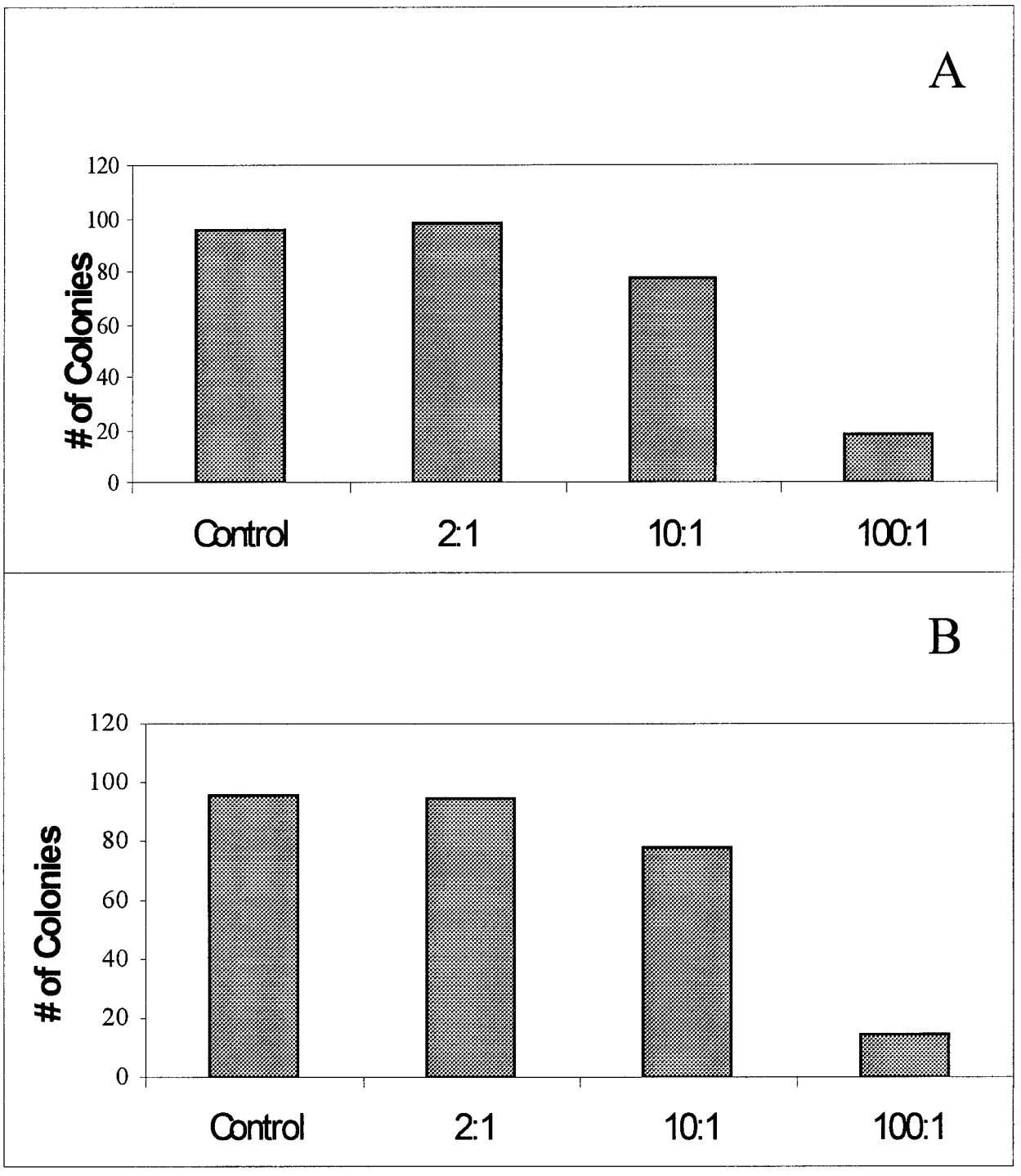


Fig. 8. MCF-7 cells were seeded into 6-well plates at 100 cells per well. 24hrs. post seeding, hypodense (A) and hyperdense (B) eosinophils were added to the wells at E:T ratios of 2:1, 10:1 and 100:1. All plates were incubated for 10 days at 37°C, 5% CO_2.

Fig.9 Growth Inhibition of MCF-7 Colony Formation by Eosinophil Cell Lines

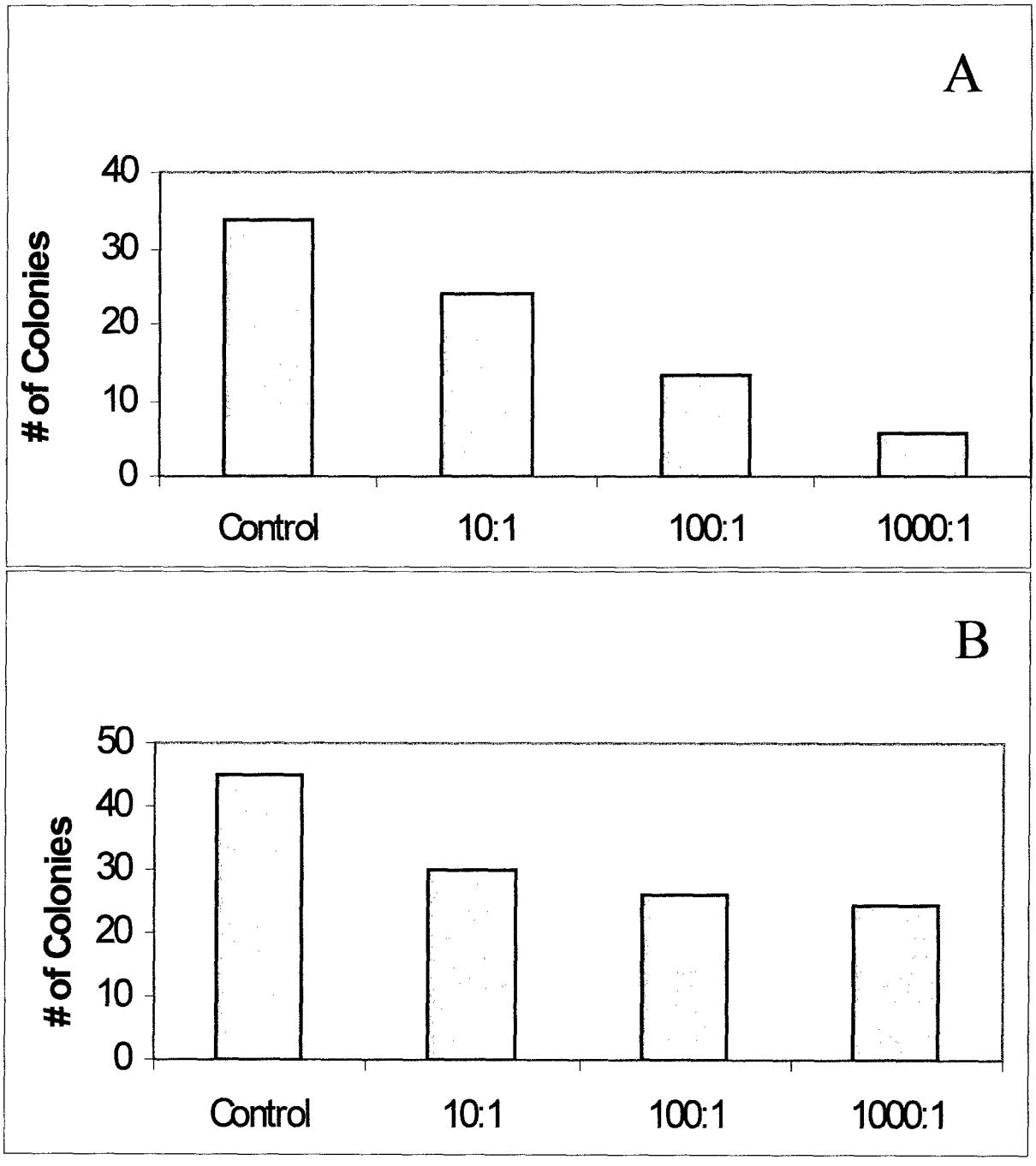
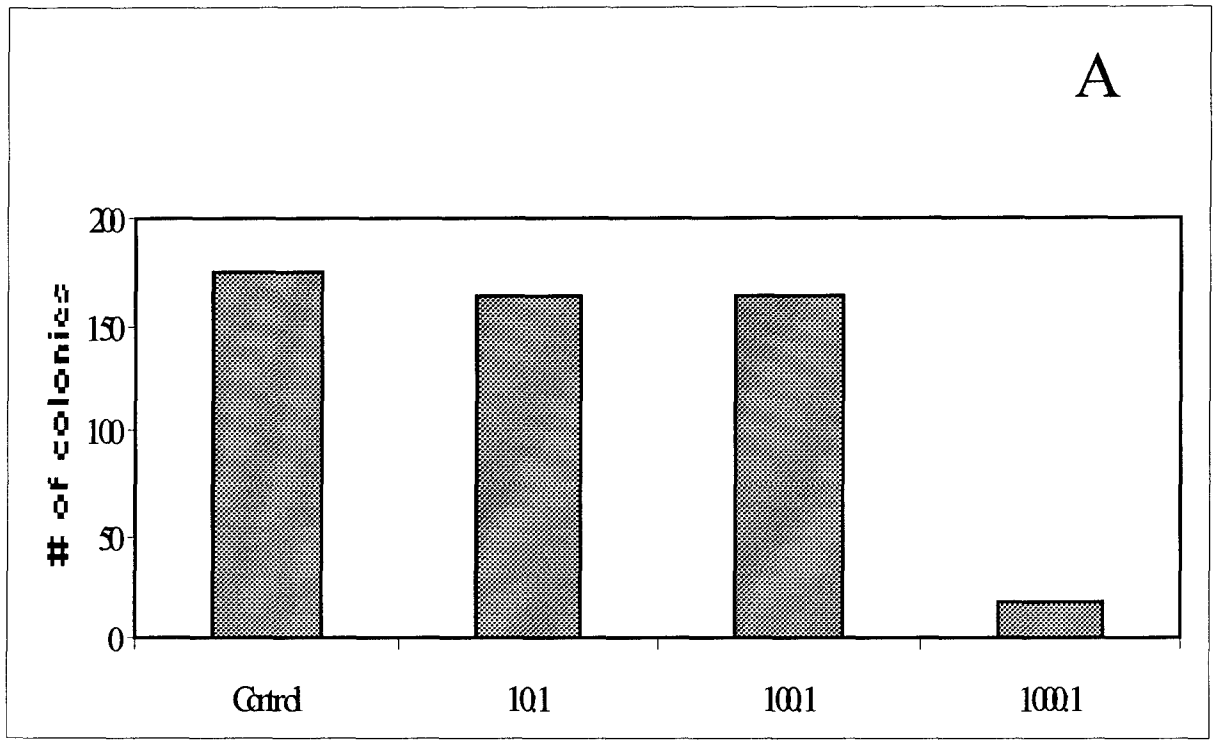


Fig. 9. BJA.060.22 (A) when cultured with MCF-7 cells at E:T ratios at 10:l, 100:l and 1000:l, inhibited colony formation dose dependently, while BJA.060.24 (B) did not.

Fig.10 Growth Inhibition of MDA-MB-231 Tumor Cell Colony Formation by Eosinophil Cell Lines



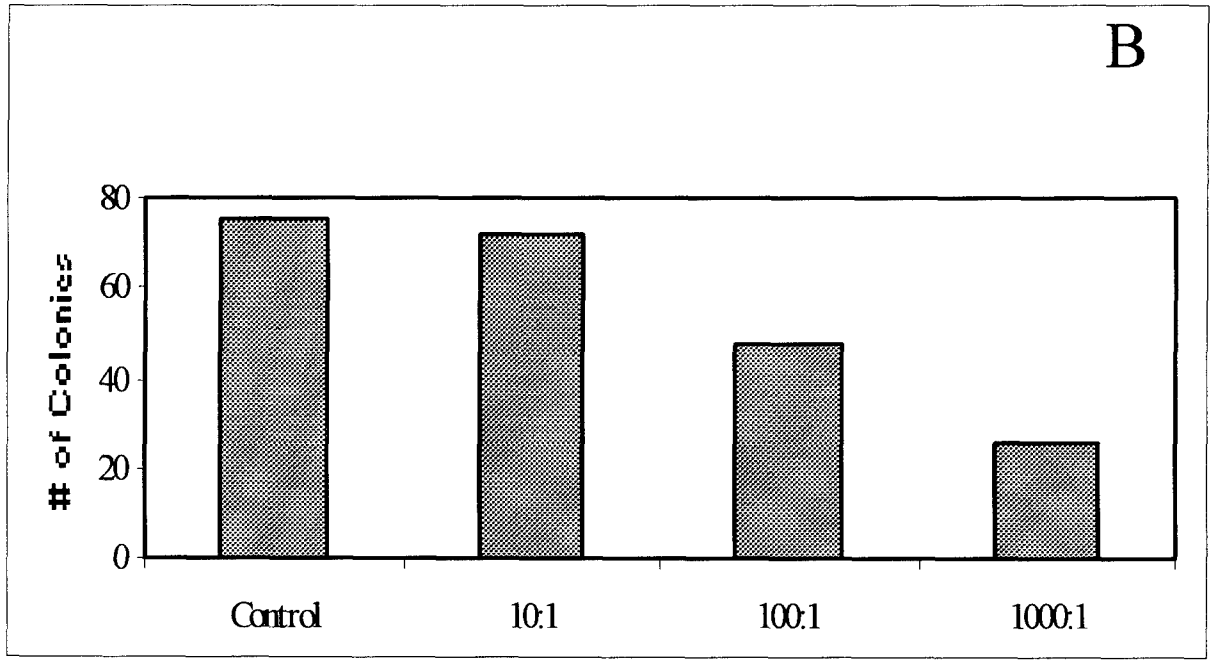


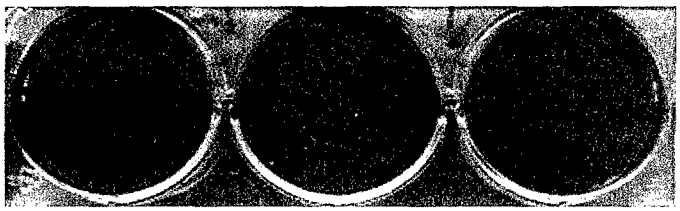
Fig.10. BJA.060.24 (B) when cultured with MDA-MB-231 tumor cells at E:T ratios of 10:1, 100:l and 1000:l inhibited colony formation in a dose dependent manner, while BJA.060.24 markedly inhibited colony formation (93%) at the E:T ratio of 1000:l.

Table 1. CYTOKINE CONCENTRATIONS IN 24HR EOSINOPHIL CULTURE SUPERNATANTS (pg/ml)

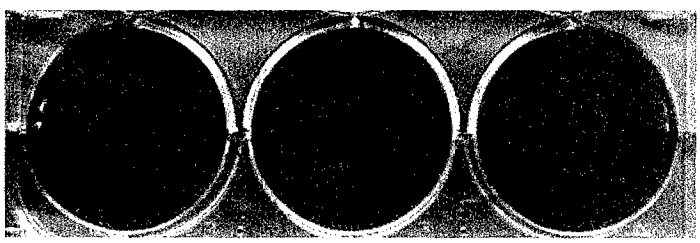
Donor	IL-4		IL-5		TNFα		GM-CSF	
	22	24	22	24	22	24	22	24
1	>1000	>1000	440	435	50	63	0	0
2	316	3	0	0	100	56	0	0
3	>1000	631	0	0	50	16	0	0
4	>1000	0	nt	nt	129	200	nt	nt
5	200	20	0	0	100	224	nt	nt
6	8	>1000	0	186	10	7.9	450	450

Table 1. 24hr. conditioned supernatants were tested for cytokines IL-4, IL-5, TNF-α and GM-CSF using commercial enzyme linked immunoassay kits.

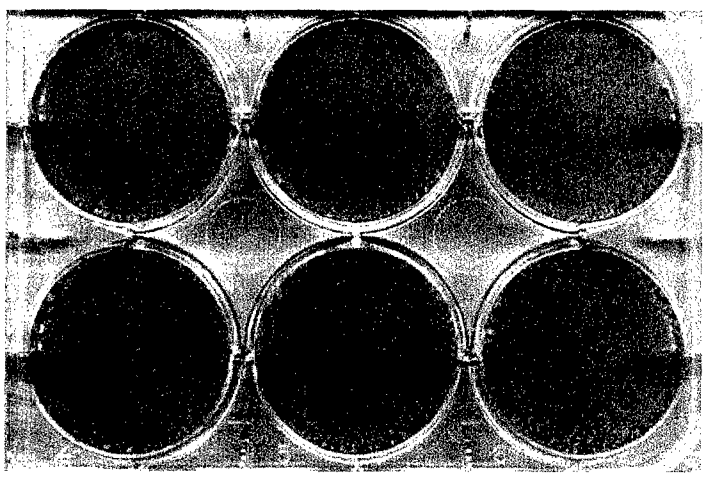
Fig.11 Eosinophil Cytotoxic Activity on MRC-5 Fibroblast Cell Growth



Control - MRC-5



GRC.014.24.S1 (1:1)

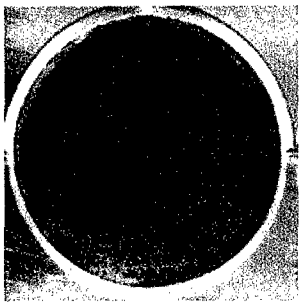


GRC.014.24.S1 (2:1)

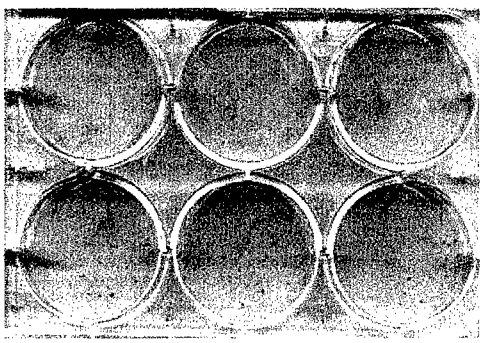
GRC.014.24.S1 (5:1)

Fig. 11. MRC-5 human lung fibroblasts were seeded into the wells of a 6-well plate at 1.5 x 10^5 cells/well and incubated overnight at 37◦C. The eosinophil subline GRC.014.24S1 was then added to the monolayers at effector to target (E:T) ratios of l:l, 2:1 and 5:l. RPMI complete medium containing 10% fetal bovine serum was added to control wells. The experiments were performed in triplicate. The plates were further incubated for 48hrs. Or until the control wells were confluent. MCF-7 tumor cells, (100cells/well) with media, conditioned supernatants or with anti-IL-4 and supernatants for 10 days after which the cells were washed 3X with PBS, then stained with hematoxylin and eosin.

Fig.12 Eosinophil Cytotoxic Activity on MCF-7 Cell Growth

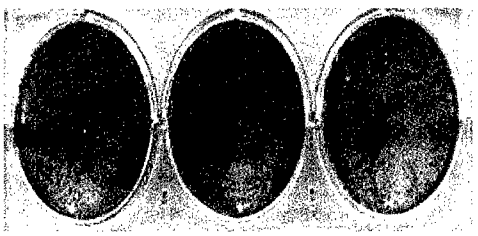


Control - MCF-7

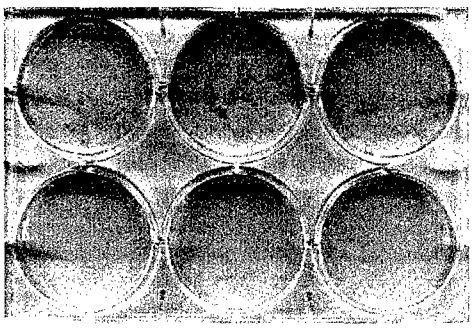


GRC.014.22.S (1:1)

GRC.014.22.S (2:1)



GRC.014.24.S1 (1:1)

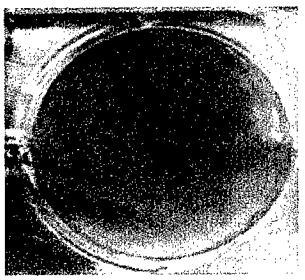


GRC.014.24.S1 (2:1)

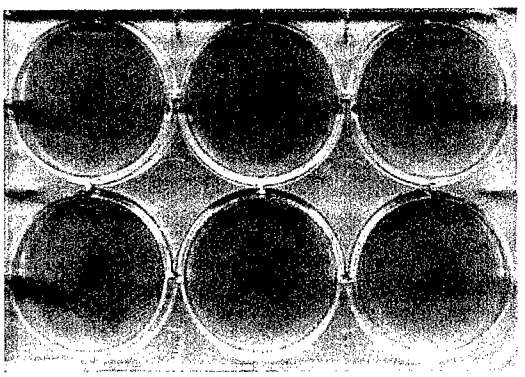
GRC.014.24.S1 (5:1)

Fig. 12. MCF-7 tumor cells were seeded into the wells of 6-well cluster culture plates at l:5 x 10^5 cells/well and incubated overnight. Eosinophil cell lines GRC.014.22S and GRC.014.24Sl at E:T ratios of l:l, 2:l and 5:l. The plates were incubated for an additional 48hr. Or until the control wells were confluent.

Fig.13 Eosinophil Cytotoxic Activity on MDA MB 231 Cell Gowth

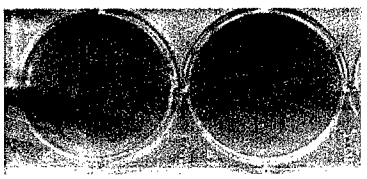


Control - MDA MB 231

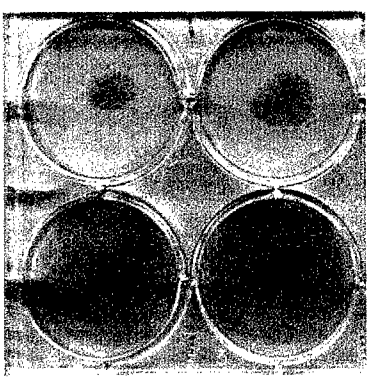


GRC.014.22.S (1:1)

GRC.014.22.S (2:1)



GRC.014.24.S1 (1:1)



GRC.014.24.S1 (2:1)

GRC.014.24.S1 (5:1)

Fig. 13. MDA tumor cells were seeded into the wells of 6-well cluster culture plates at l.5 x 10^5 cells/well and incubated overnight. Eosinophil cell lines GRC.014.22S and GRC.014.24Sl at E:T ratios of l:l, 2:l and 5:l. The plates were incubated for an additional 48hr or until the control wells were confluent..

Fig. 14.

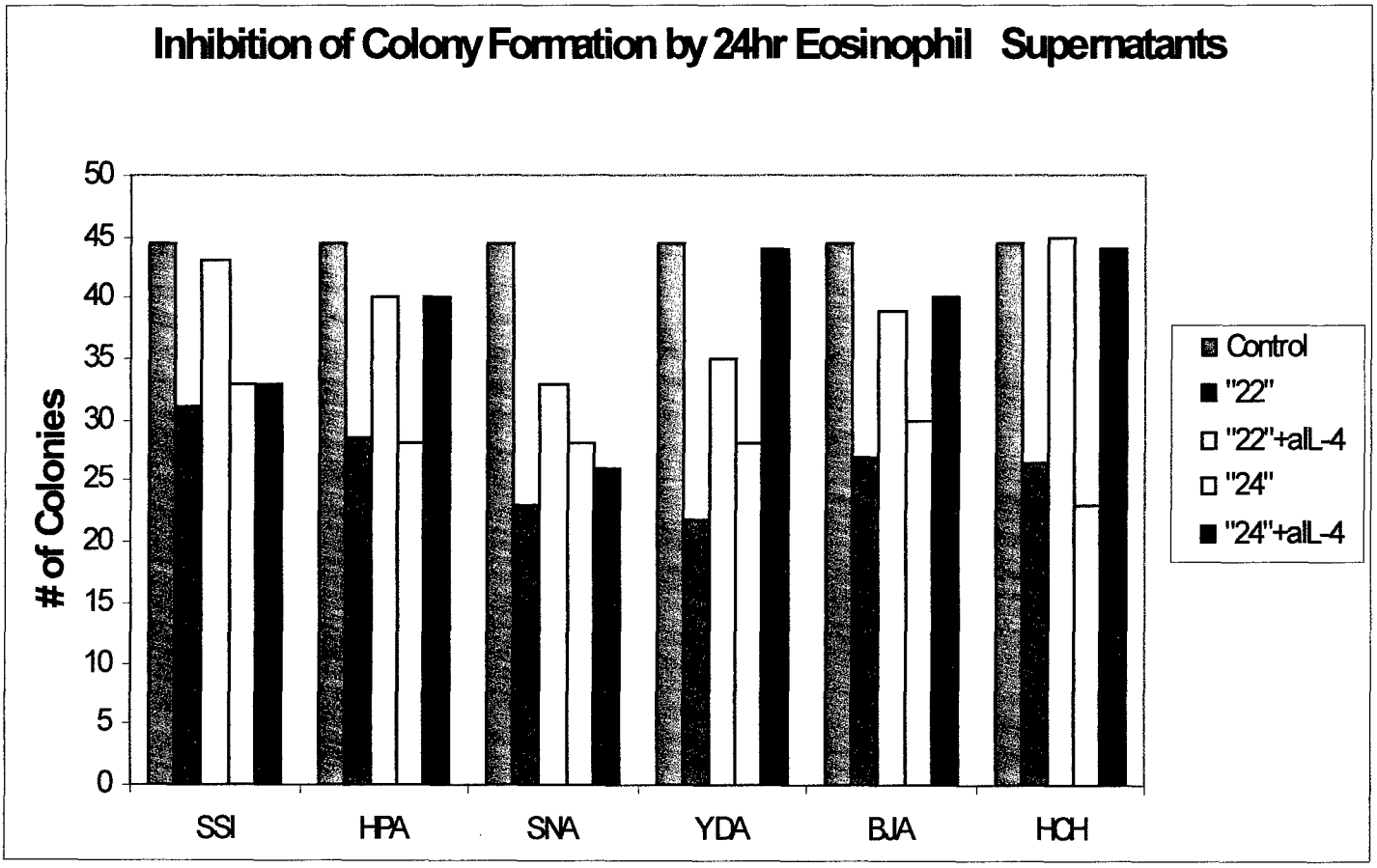

Inhibition of Colony Formation by 24hr Eosinophil Supernatants
# of Colonies
50
45
40
35
30
25
20
15
10
5
0
SSI
HPA
SNA
YDA
BJA
HCH
Control
"22"
"22"+aIL-4
"24"
"24"+aIL-4

Fig. 14. MCF-7 tumor cells, (100cells/well) with media, conditioned supernatants or with anti-IL-4 and supernatants for 10 days after which the cells were washed 3X with PBS, then stained with hematoxylin and eosin.

Fig.15 GRC.014.22 Eosinophil Supernatants Inhibit MCF-7 Colony Formation In Vitro

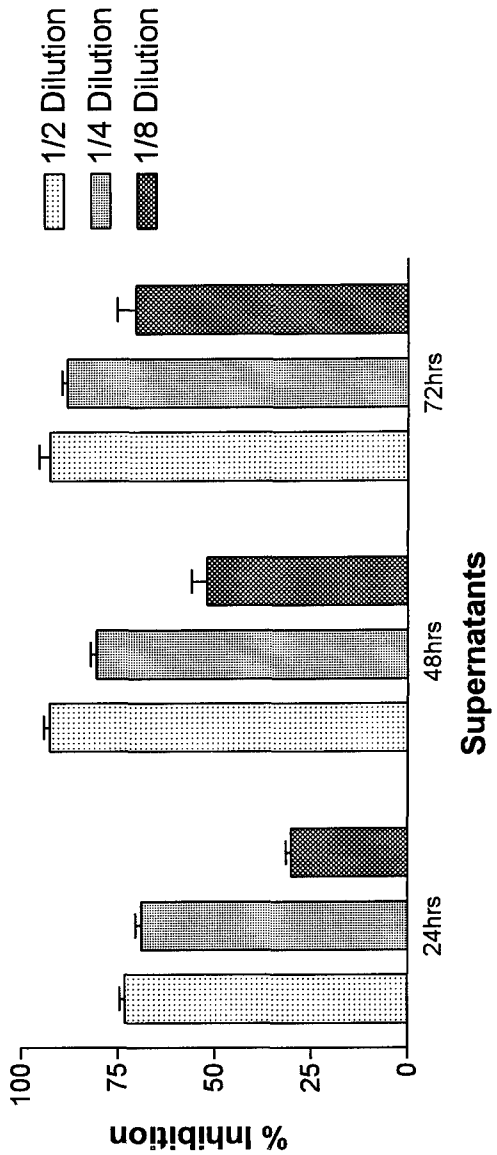


Fig. 15. MCF-7 tumor cells were seeded into wells of a 6-well plate at 100 cells per well and incubated overnight. The cells were then incubated with cell free supernatants from eosinophil cell line GRC.014.22 cultures for 10 days at 37°C. Plates were washed 3x with phosphate-buffered saline; stained with H & E, then counted.

Fig.16 GRC.014.22 Eosinophil Supernatants Inhibit MDA MB 231 Colony Formation In Vitro

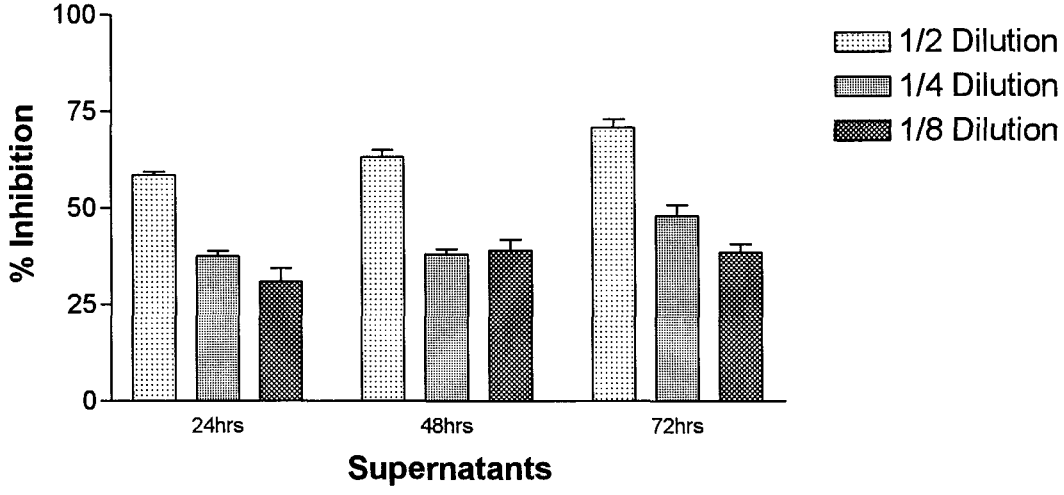


Fig. 16. MDA-MB-231 tumor cells were treated GRC.014.22 supernatants similarly to that described for MCF-7 in fig. 15. The colonies were stained and counted. Percent inhibition was determined.

Fig.17 The Effect of GRC.014.24 Eosinophil Cultured Supernatants on MCF-7 Breast Tumor Cells

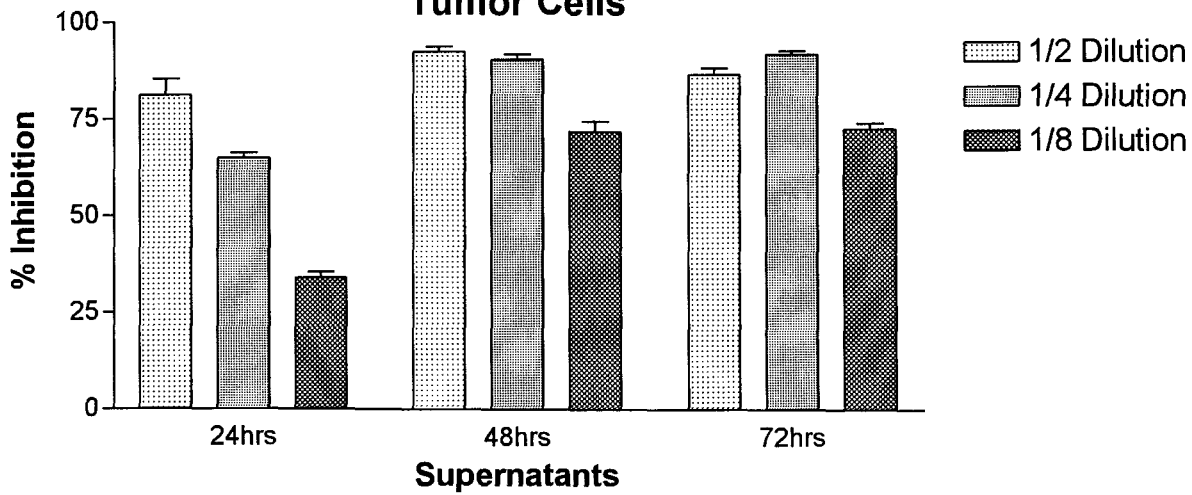


Fig.18 The Effect of GRC.014.24 Eosinophil Cultured Supernatants on MDA MB 231 Colony Formation

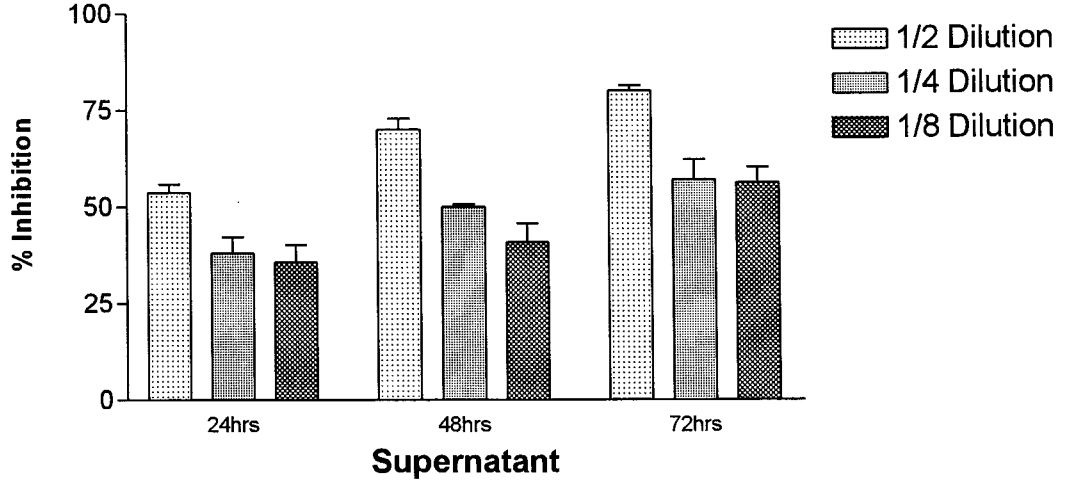


Fig. 17, 18. Cultured supernatants from GRC.014.24 eosinophils were examined for inhibitory activity against MCF-7 and MDA-MB-231 colony formation.

Fig.19 Effect of SD.031.22 Eosinophil Cultured Supernatants on MCF-7 Colony Formation

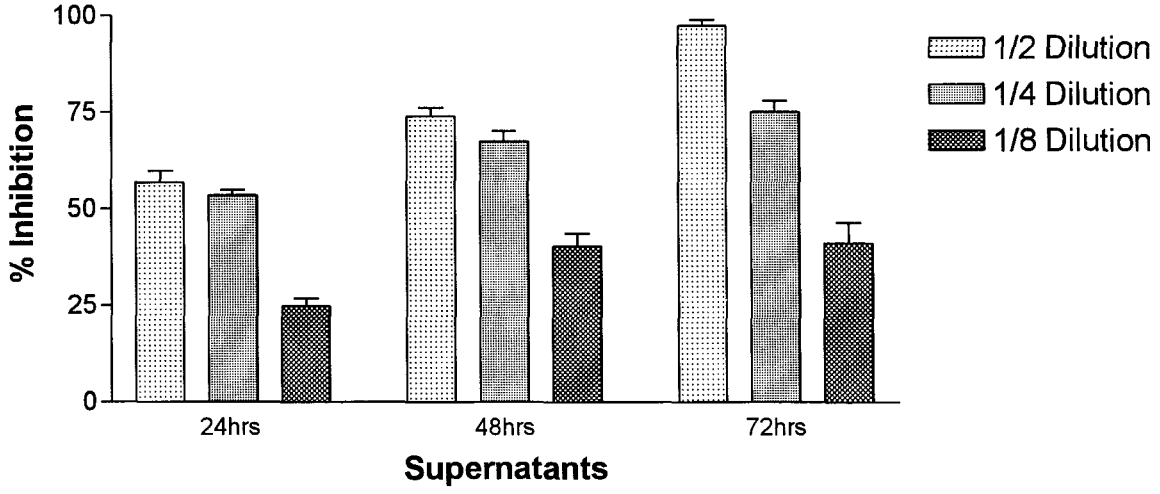


Fig.20 Effect of SD.031.22 Eosinophil Cultured Supernatants on MDA MB 231 Colony Formation

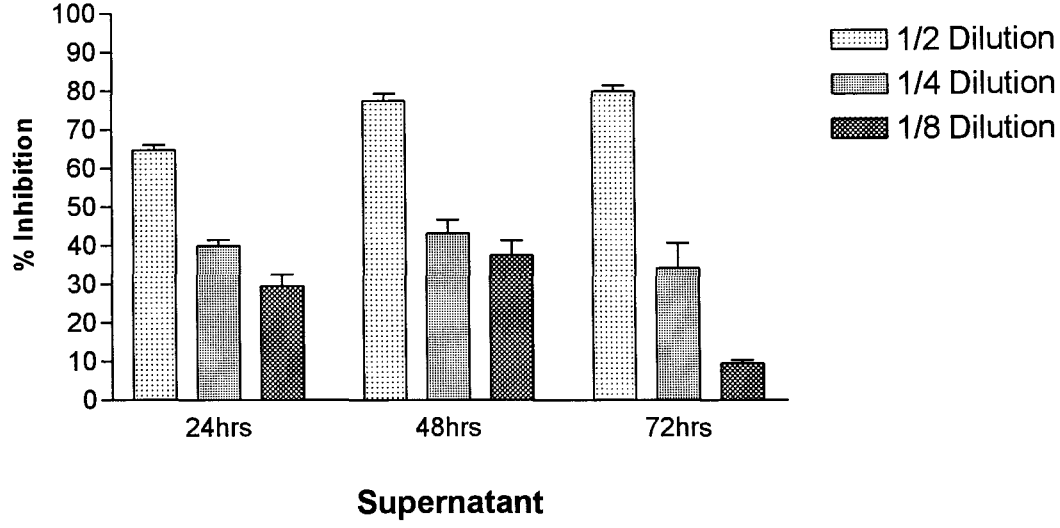


Fig 19, 20. Cultured supernatants from eosinophil cell line SD.031.22 were incubated with MCF-7 and MDA-MB-231 tumor cells. The cells were incubated for 10 days as described previously. After harvesting, and staining, the colonies were counted and the % inhibition determined.

Fig.21 Effect of SD.031.24 Eosinophil Cultured Supernatants on MCF-7 Colony Formation

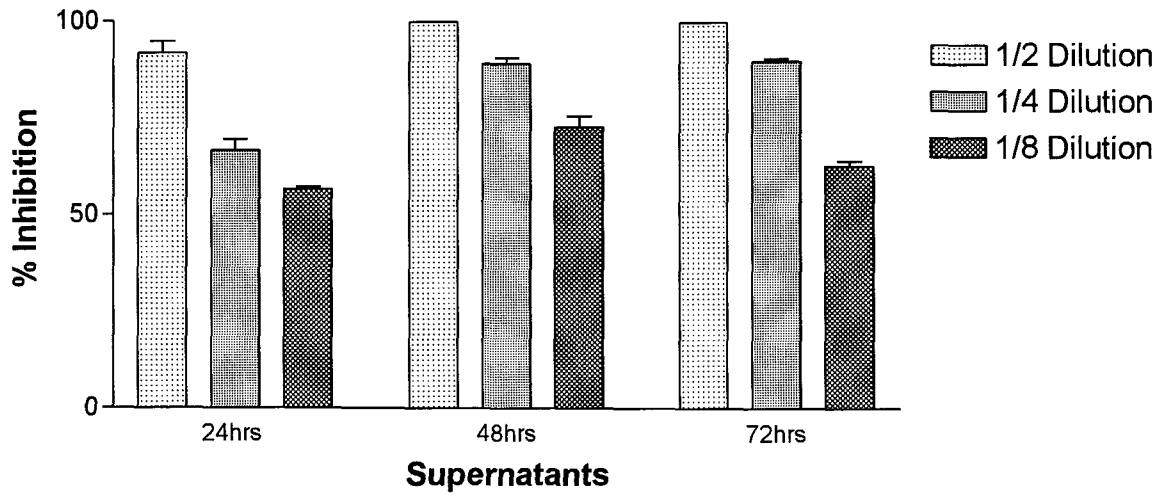


Fig.22 Effect of SD.031.24 Eosinophil Cultured Supernatants on MDA MB 231 Colony Formation

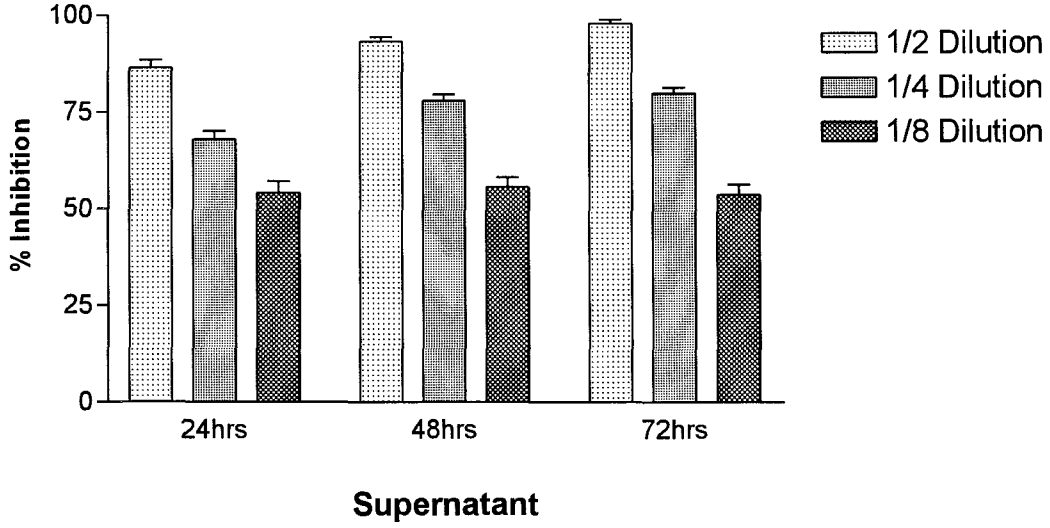


Fig. 21, 22. MCF-7 (Fig 21) and MDA-MB-231 tumor cells were seeded into 6-well plates (l00 cells/well) and incubated overnight. Control wells contained RPMI complete medium with 10% FBS. Serial toe-fold dilutions of cultured supernatants from the eosinophil cell line SD.031.24 were added and the plates were incubated for an additional l0 days. Colonies were stained with H & E and counted. Percent inhibition was determined.

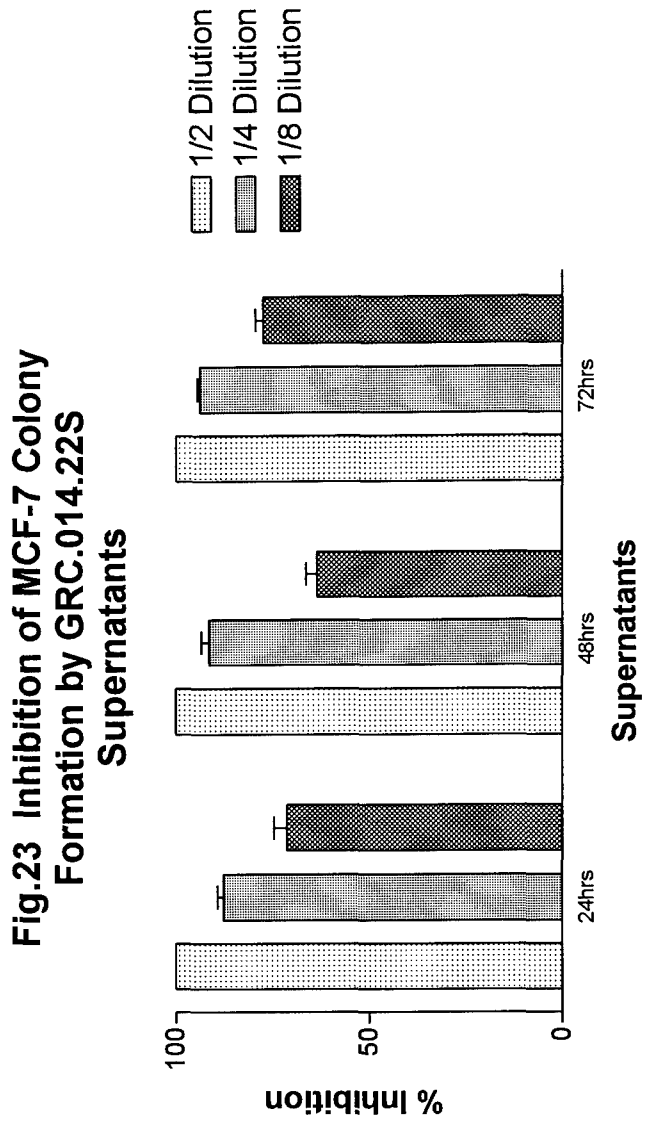


Fig.23 Inhibition of MCF-7 Colony Formation by GRC.014.22S Supernatants

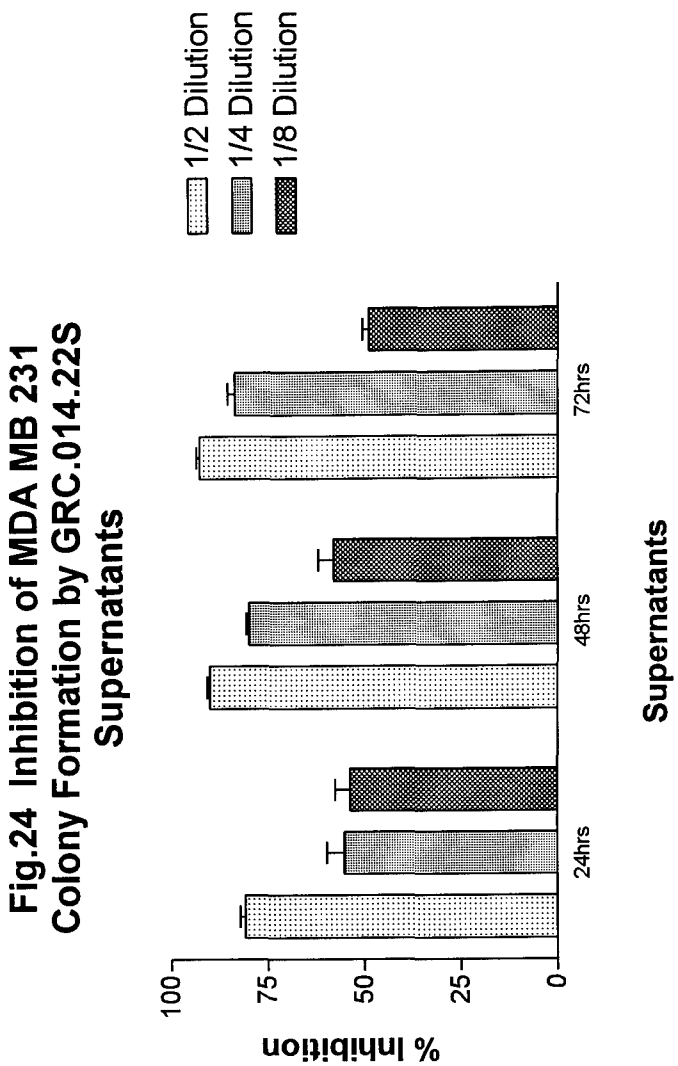


Fig.24 Inhibition of MDA MB 231
Colony Formation by GRC.014.22S
Supernatants
1/2 Dilution
1/4 Dilution
1/8 Dilution
% Inhibition
100
75
50
25
0
24hrs
48hrs
72hrs
Supernatants

Fig. 23, 24. Supernatants from the eotaxin receptor positive subline GRC.014.22S were incubated with tumor cells MCF-7 and MDA-MB-231 (l00 cell/well) in 6-well plates as described previously. Percent inhibition of colony formation was determined.

Fig.25 Cultured Supernatants from an Eotaxin Positive (CCR3+) Eosinophil Cell Line (GRC.014.24S) Inhibit MCF-7 Colony Formation

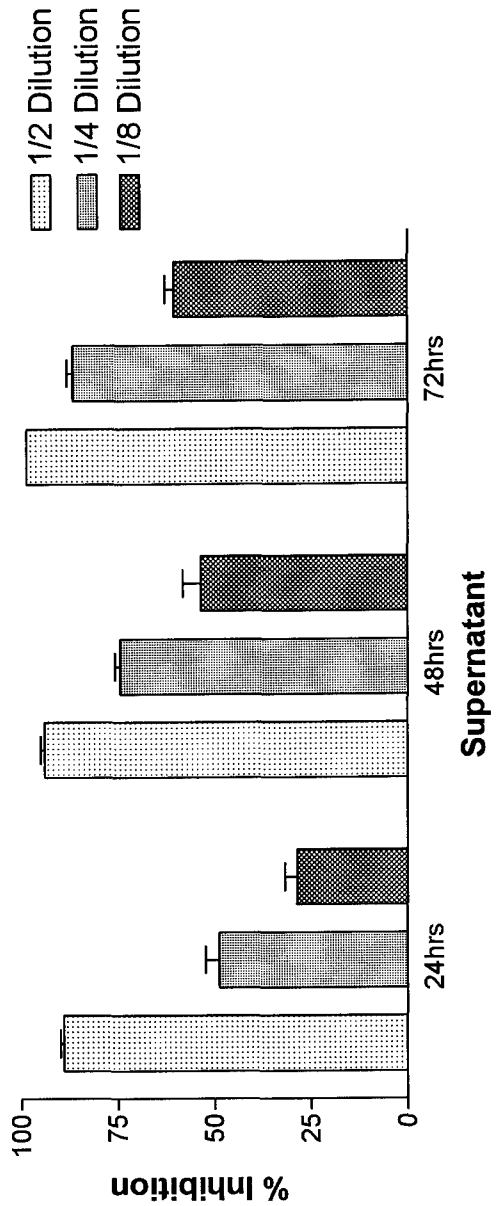


Fig.26 Cultured Supernatants from an Eotaxin Positive (CCR3+) Eosinophil Cell Line (GRC.014.24S) Inhibit MDA MB 231 Colony Formation

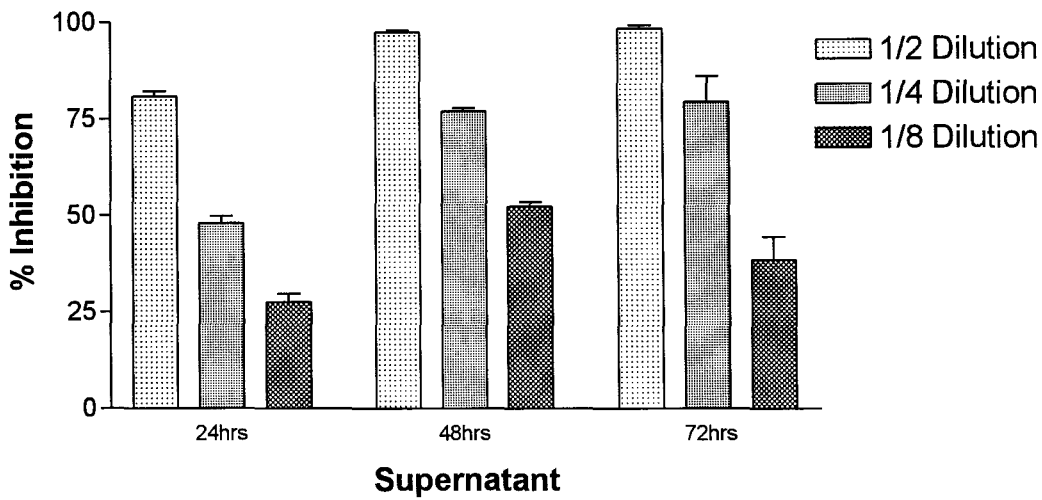


Fig. 25, 26. Cultured supernatants from the CCR3^{+} eosinophil line GRC.014.24S similarly to that described in figures 23 and 24.

Fig.27 Cultured Supernatants from CCR3+, CD49d+ Eosinophil Cell Line Inhibit MCF-7 Colony Formation

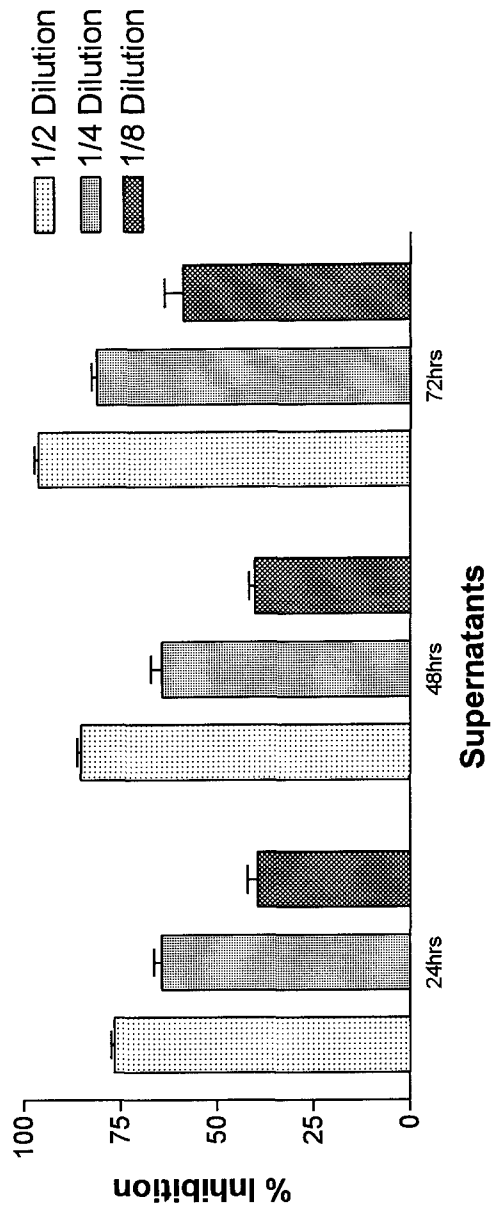


Fig.28 Cultured Supernatants from a CCR3+, CD49d+ Eosinophil Cell Line Inhibit MDA MB 231 Colony Formation

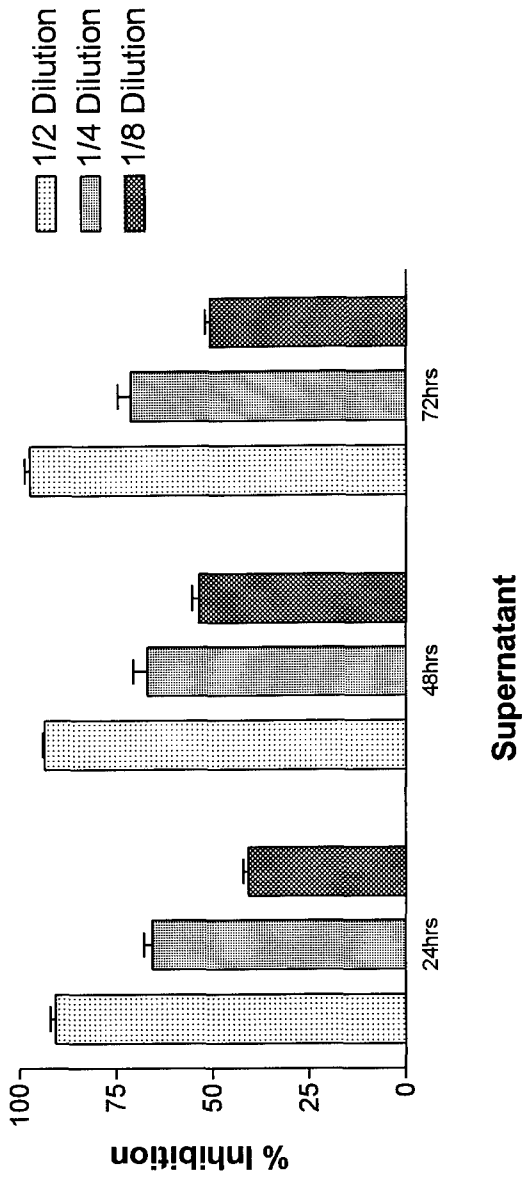


Fig. 27, 28. Cultured supernatants from $CCR3^+$ cells that were sorted using the CD49d marker, were incubated with MCF-7 and MDA-MB-231 tumor cells similarly to that described in figure 23 and 24.

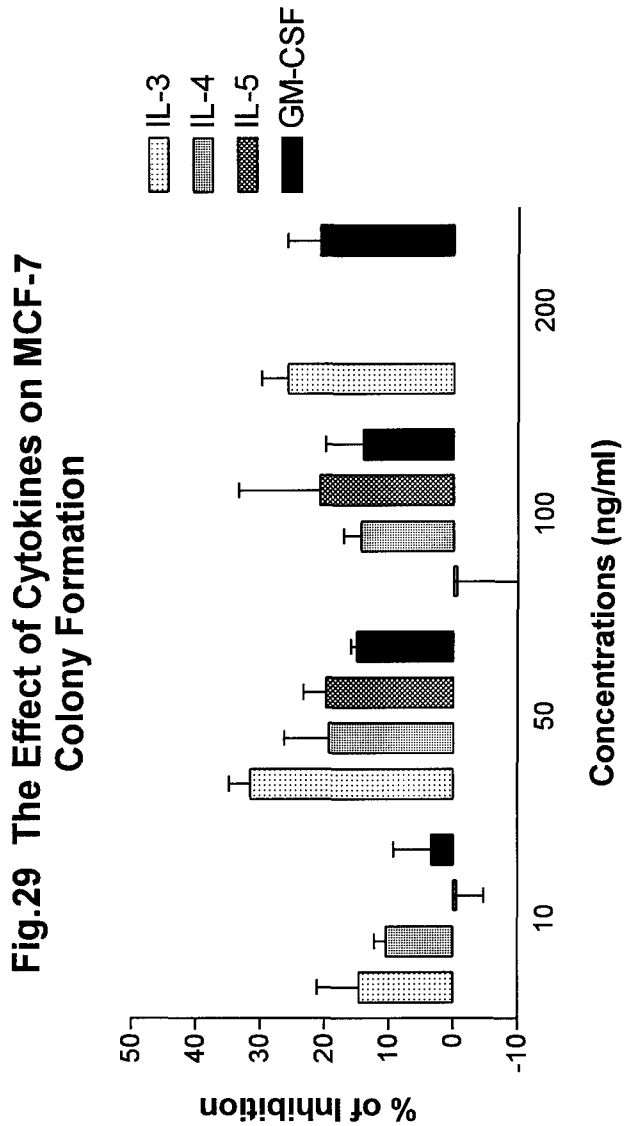


Fig.29 The Effect of Cytokines on MCF-7 Colony Formation

Fig. 29. MCF-7 cells were seeded into 6-well plates (100 cell/well), then treated with cytokines IL-3, IL-4, IL-5, GM-CSF at 10, 50, 100 and 200ng/ml. The plates were incubated for 10 days; harvested; colonies stained then counted.

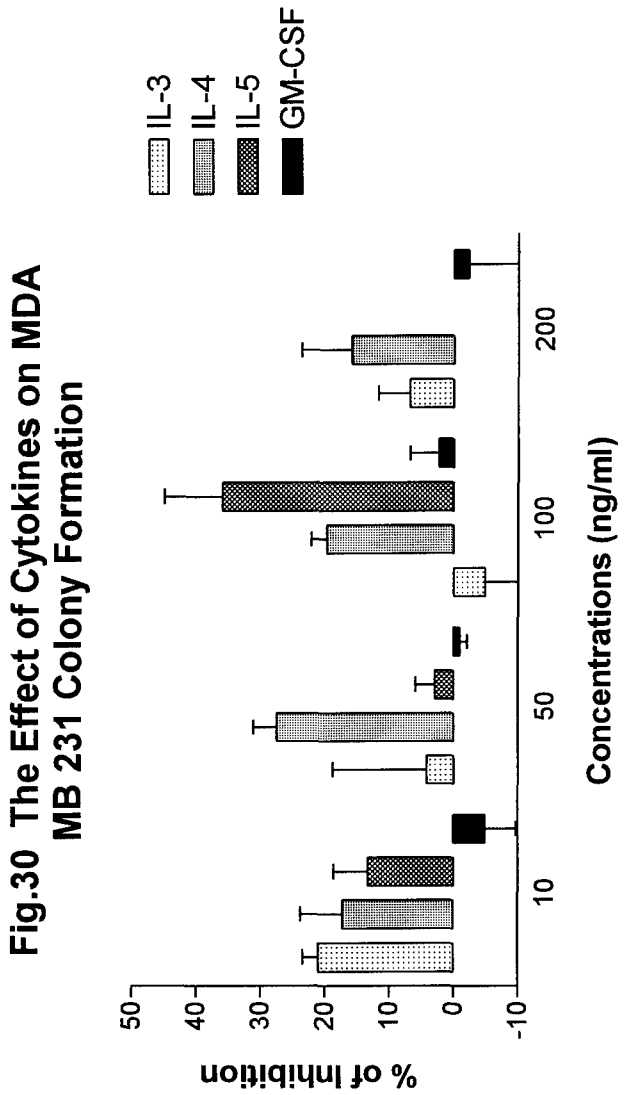


Fig.30 The Effect of Cytokines on MDA MB 231 Colony Formation
IL-3
IL-4
IL-5
GM-CSF
% of Inhibition
50
40
30
20
10
0
-10
10
50
100
200
Concentrations (ng/ml)

Fig. 30. MDA-MB-231 tumor cells were seeded; incubated with cytokines and examined for colony formation as described in fig. 28.

Fig.31 Effect of TNF-a on MCF-7 Colony Formation

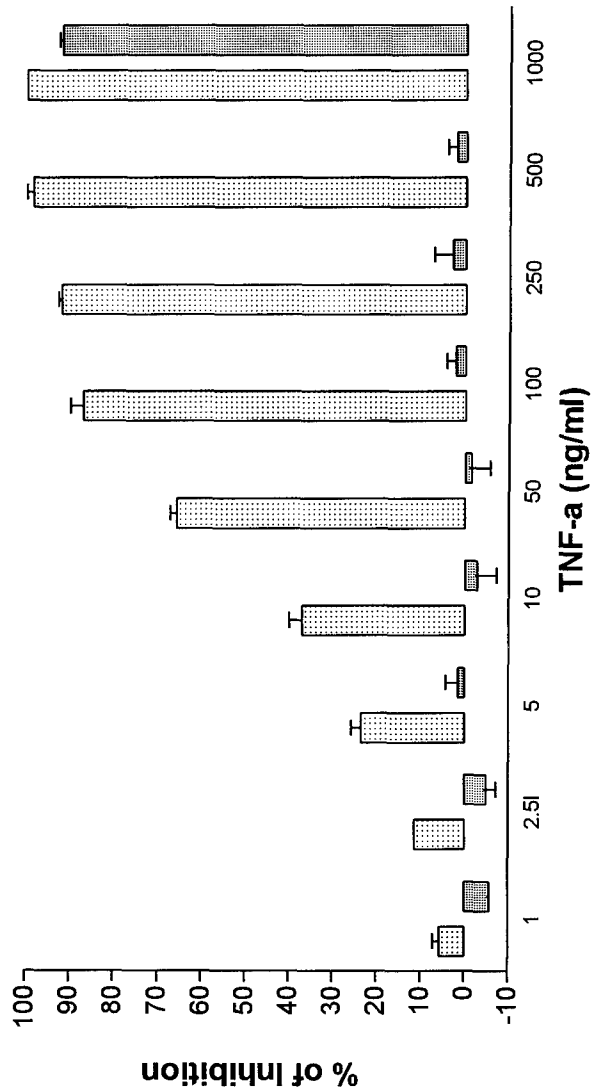


Fig. 31. MCF-7 tumor cells were seeded into wells of 6-well plate at 100 cells/well and treated with different concentrations of TNFα, (200ul/well. Each concentration and media control was set up in triplicate. Anti-TNFα (100ng/ml) was also added to certain wells. The plates were further incubated for 10 days.

Fig.32 Inhibitory Effect of Eosinophil Cell Line on MRC-5 Fibroblast Cell Growth

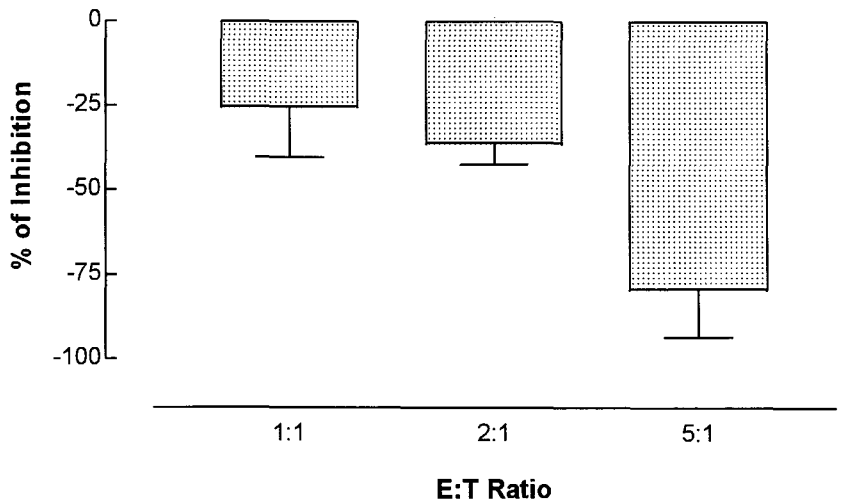


Fig. 32. MRC-5 Fibroblast cells were seeded into the walls of 6-well cluster plates at l.5 x 10^5 cells/well and incubated overnight. Sorted eosinophil cell lines were added at different E:T ratio in duplicates. The plates were incubated for an additional 72hrs, rinsed 3x with PBS, stained with H& E and the ID's were measured. The percentage of inhibition was determined against the assay control.

Fig.33 Inhibitory Effect of Eosinophil Sublines on MCF-7 Growth

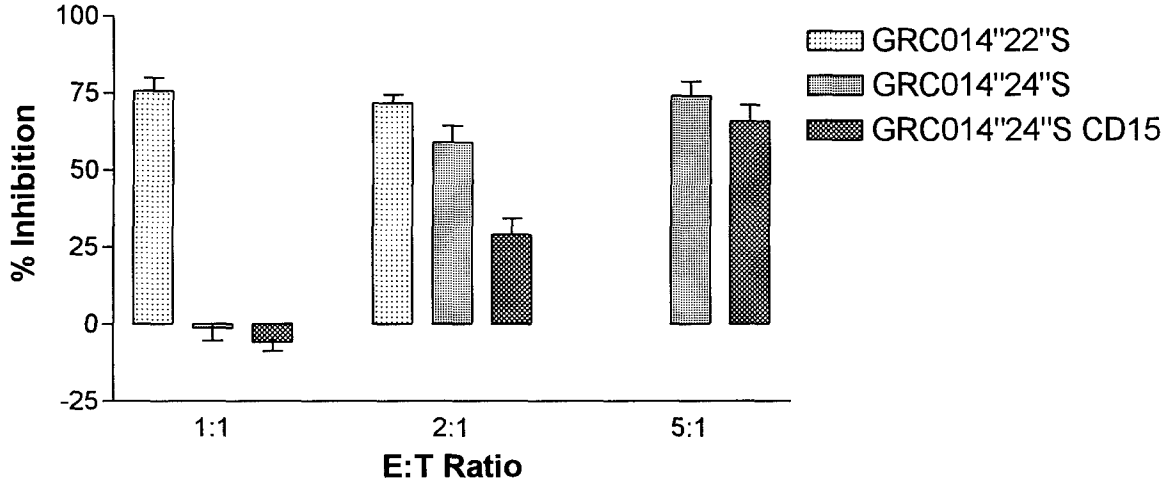


Fig. 33. MCF-7 tumor cells were seed into the walls of 6-well cluster culture plates at l.5 x 10^5 cells/well and incubated overnight. Sorted eosinophil cell lines were added at different E:T ratio in duplicates. The plates were incubated for an additional 72hrs, rinsed 3x with PBS, stained with H& E and the ID's were measured. The percentage of inhibition was determined against the assay control.

Fig.34 Inhibitory Effect of Eosinophil Sublines on MDA-MB-231 Growth

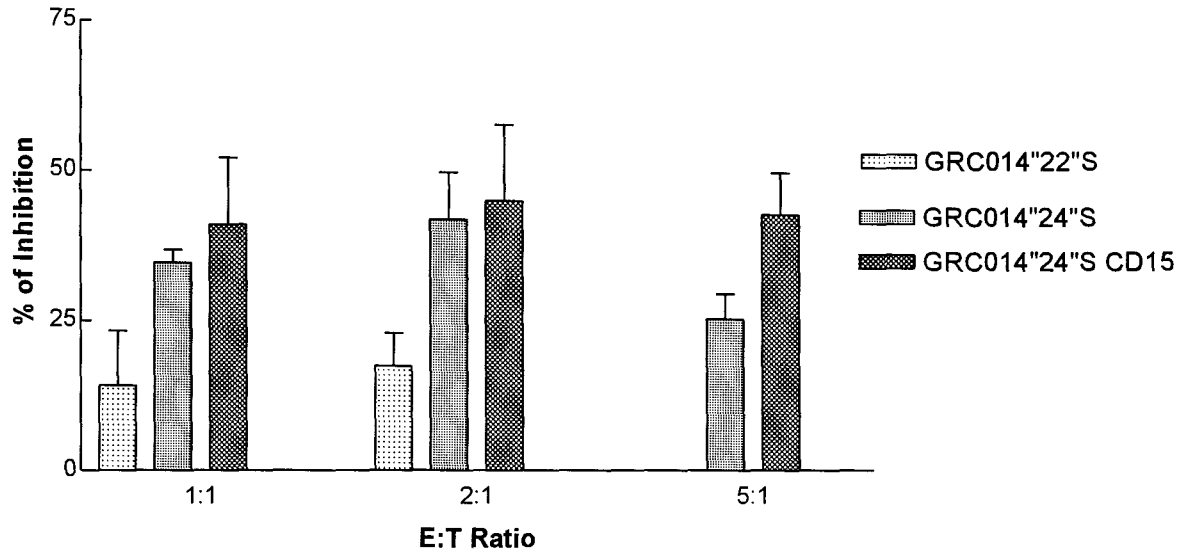


Fig. 34. MDA-MB-231 tumor cells were seeded into the walls of 6-well cluster culture plates at 1.5 x 10^5 cells/well and incubated overnight. Sorted eosinophil cell lines were added at different E:T ratio in duplicates. The plates were incubated for an additional 72hrs, rinsed 3x with PBS, stained with H&E and the ID's were measured. The percentage of inhibition was determined against the assay control.

Fig.35 The Effect of Peripheral Blood Eosinophils on MCF-7 Cell Growth

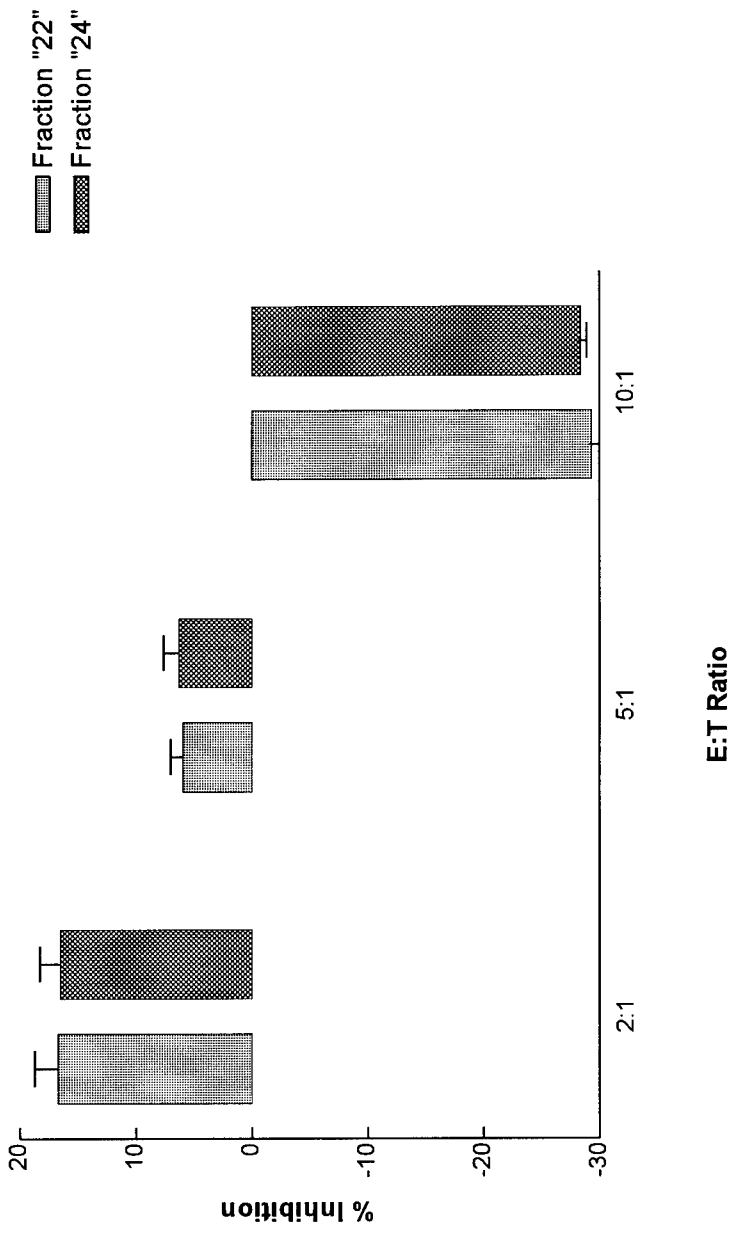


Fig. 35. MCF-7 tumor cells were added into the walls of 6-well cluster culture plates at 1.5 x 10^5 cells/well and incubated overnight. Fresh eosinophil metrizamide gradient fractions were added in triplicates at different E:T ratio and incubated for an additional 72hrs. Wells were washed 3x with PBS, stained with H& E and the IDV's recorded. The percentage of inhibition was determined against the control average value.

Fig.36 The Effect of Peripheral Blood Eosinophils on MDA-MB-231

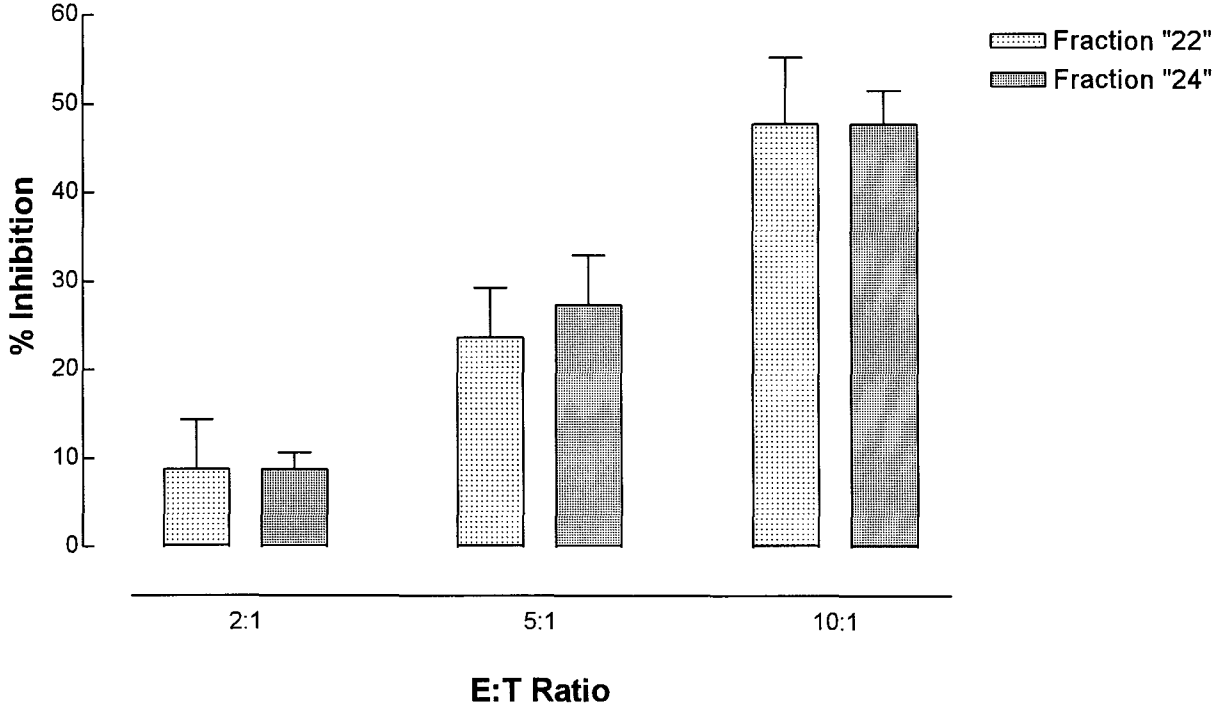


Fig. 36. MDA-MB 231 tumor cells were added into the walls of 6-well cluster culture plates at 1.5 x 10^5 cells/well and incubated overnight. Fresh eosinophil metrizamide gradient fractions were added in triplicates at different E:T ratio and incubated for an additional 72hrs. Wells were washed 3x with PBS, stained with H&E and the IDV's recorded. The percentage of inhibition was determined against the control average value.

Fig.37 The Effect s of TNF-alpha and anti-TNF-alpha on MCF-7 Cell Growth

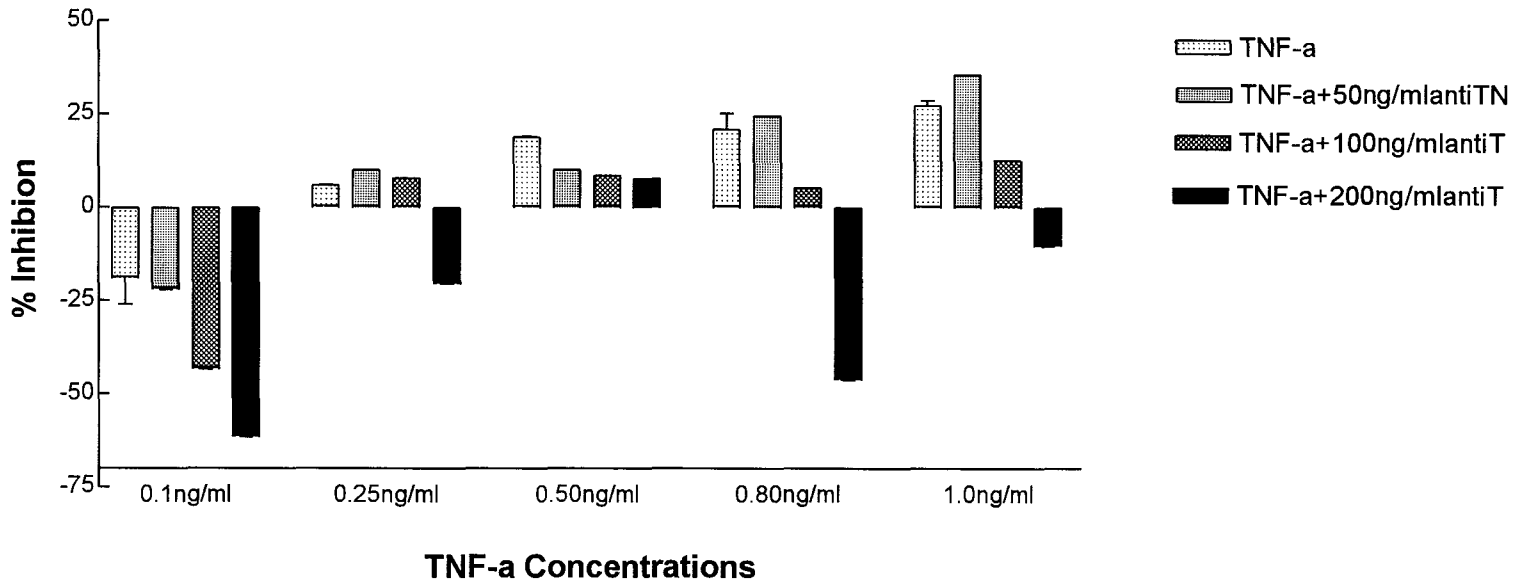


Fig. 37. MCF-7 tumor cells were seeded into the wall of 6-well cluster culture plates at 1.5 x 10^5 cells/well and incubated overnight. Cells were treated with different concentrations of TNFα in the presence of various concentrations of anti TNFα. After 72hrs of incubation, wells were washed, stained and ID recorded. Percentage of inhibition was determined using the average ID of the test control (tumor cells + media).

Fig.38 TNF-alpha is an Inhibitory Component of Eosinophil Cell Line Supernatants

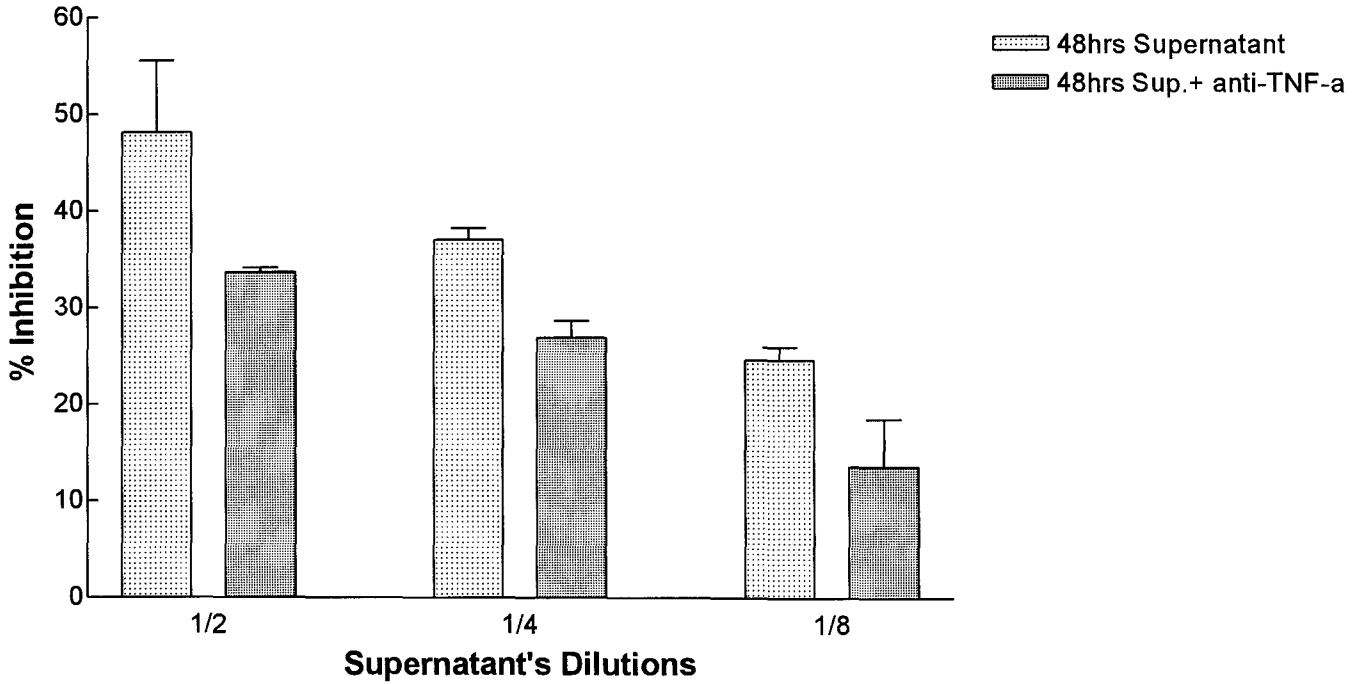


Fig. 38. MCF-7 tumor cells were seeded into the wells of 6-well cluster culture plates at 1.5 x 10^5 cells/well and incubated overnight. 48hrs supernatants (conditioned supernatants) from eosinophil cell lines were used to treat the wells at different dilutions in triplicates, for an additional 72hrs. Wells were washed 3x with PBS, stained with H&E and the ID's recorded. The percentage of inhibition was determined against the control average value.

Fig.39 TNF-alpha is an Inhibitory Component of Eosinophil Cell Line Supernatants

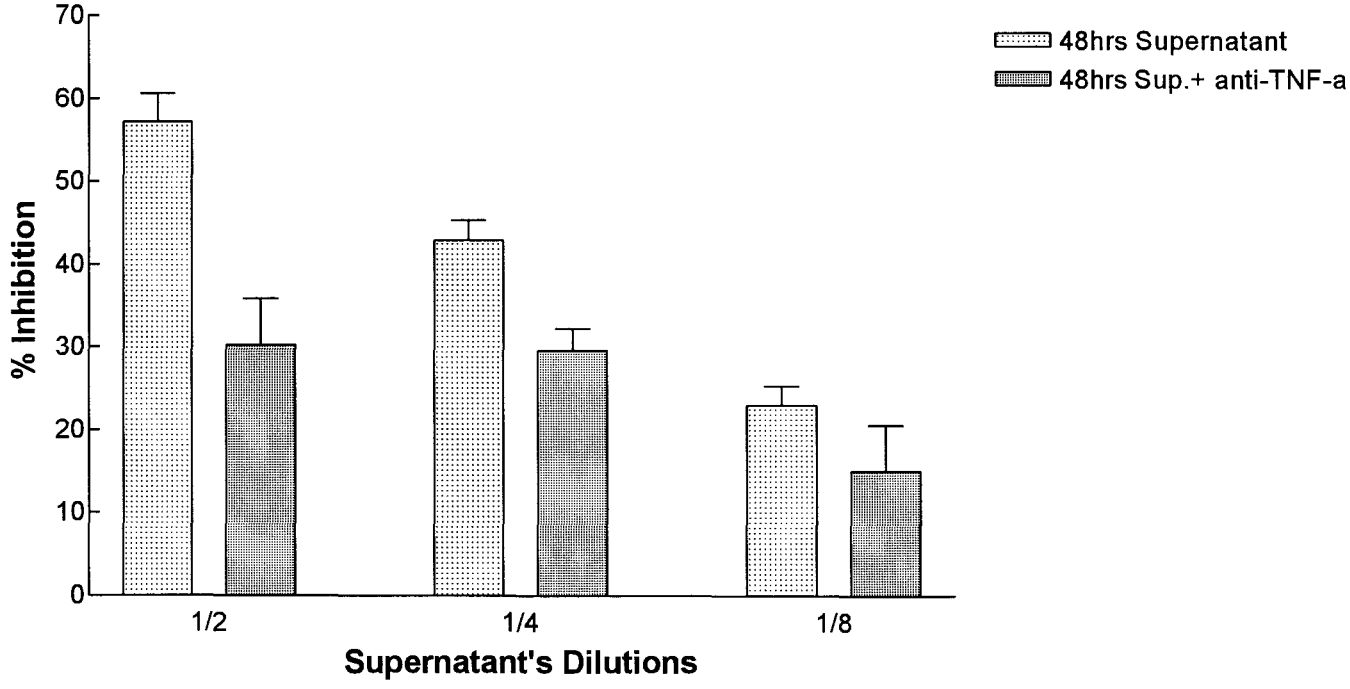


Fig. 39. MDA-MB 231 tumor cells were seeded into the wells of 6-well cluster culture plates at 1.5 x 10^5 cells/well and incubated overnight. 48hrs supernatant (conditioned supernatants) from eosinophil cell lines were used to treat the wells at different dilutions in triplicates, for an additional 72hrs. Wells were washed 3x with PBS, stained with H&E and the IDV's recorded. The percentage of inhibition was determined against the control average value.

Fig. 40. MCF-7 Multicellular Tumor Spheriods

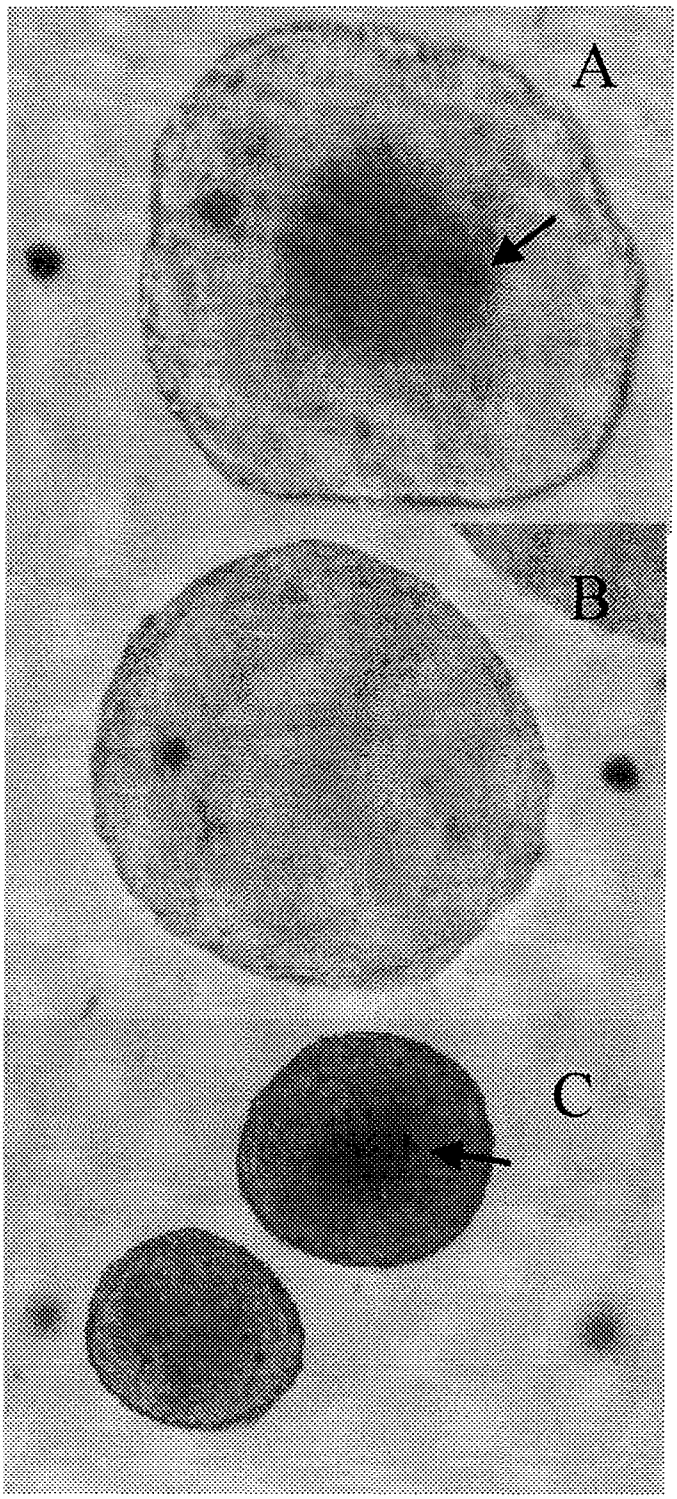


Fig. 40. MTS were developed by rocking 1×10^6 MCF-7 cells in a sealed T25 flask at 30 rpm for 48hr. At 37°C. The spheroids were then transferred to 100mm petri dishes containing 0.3% agar overlay, then cultured at 37°C, 5% CO_2 for 7-14 days.

Fig.41. Peripheral Blood Hypodense and Hyperdense Eosinophils Bind to MCF-7 Multi-cellular Tumor Spheriods

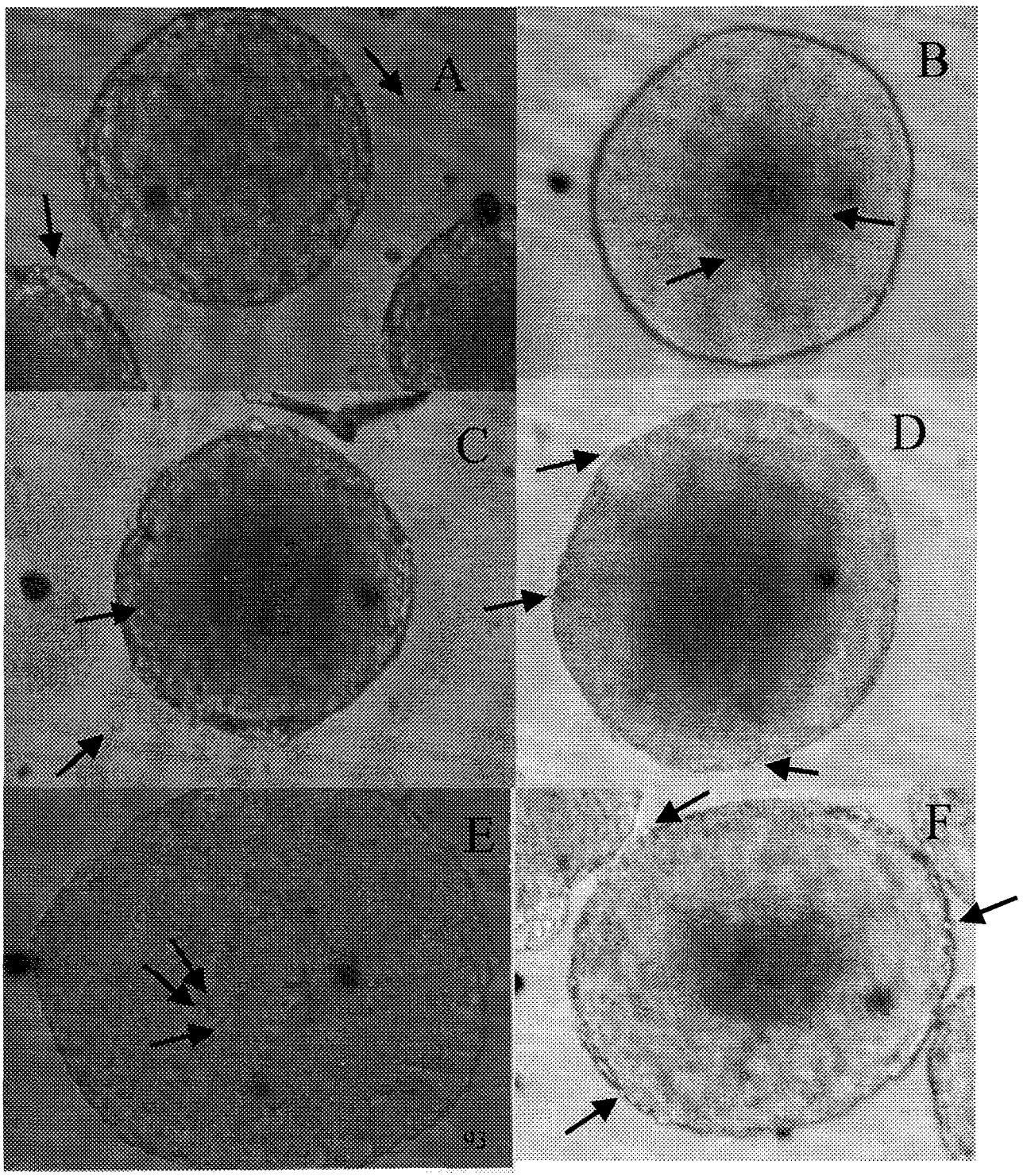


Fig. 41. Hypodense (A,C and E) and hyperdense (B,D and E) eosinophils from 3 individuals were cultured with 2 day-old MTS at an E:T ratio of 100:1 for 7 days. Arrows indicate bound eosinophils.

Fig. 42. Hypodense Eosinophilic Cell Line Binds to MCF-7 Multi-cellular Tumor Spheriods

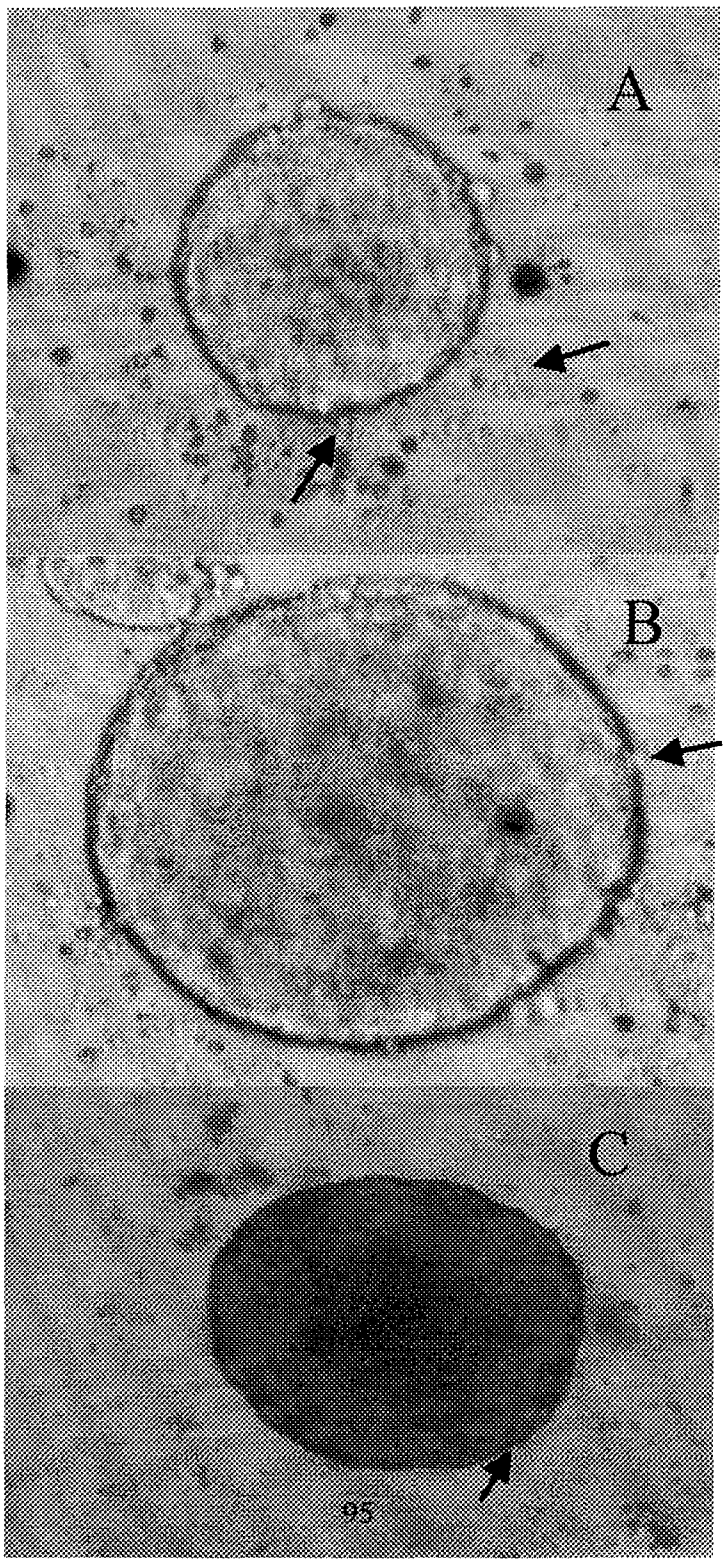
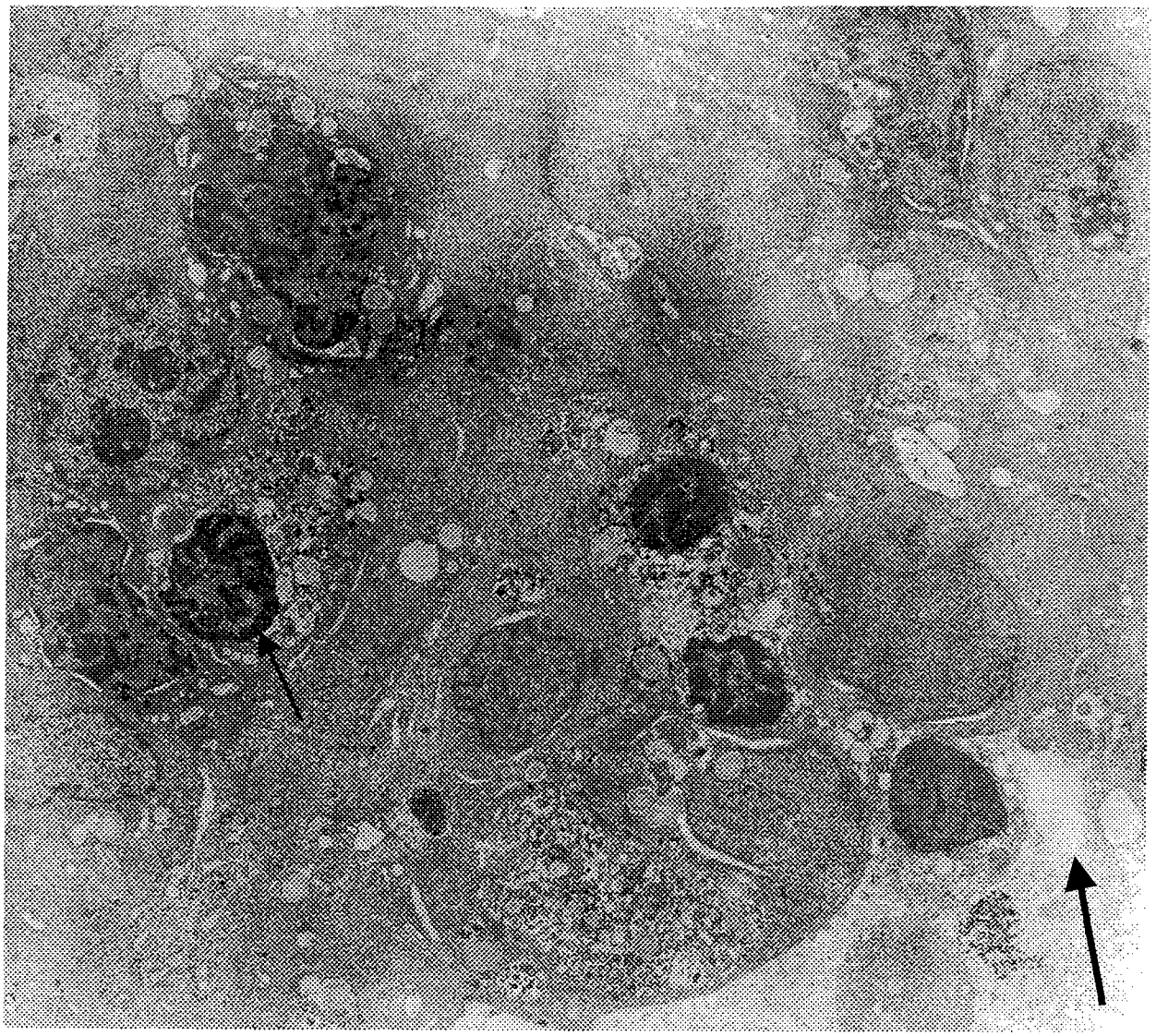


Fig. 42. Hypodense eosinophil cell line BJA.060.22 was cultured with 2-day old MCF-7 MTS at E:T ratios of 10:1 (A) 100:1 (B) and 1000:1 (C) for 7 days.

Fig. 43. Eosinophil Infiltration of MCF-7 Multicellular Tumor Spheroid



MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB263458
ADB282174
ADB270704
ADB282196
ADB264903
ADB268484
ADB282253
ADB282115
ADB263413
ADB269109
ADB282106
ADB262514
ADB282264
ADB256789
ADB251569
ADB258878
ADB282275
ADB270822
ADB282207
ADB257105
ADB281673
ADB254429
ADB282110
ADB262549
ADB268358
ADB257359
ADB265810
ADB282111
ADB273020
ADB282185
ADB266340
ADB262490
ADB266385
ADB282181
ADB262451
ADB266306
ADB260298
ADB269253
ADB282119
ADB261755
ADB257398
ADB267683
ADB282231
ADB234475
ADB247704
ADB258112
ADB267627
ADB282838
ADB233092
ADB263929
ADB282182
ADB257136
ADB282227
ADB282177
ADB263548
ADB246535
ADB282826
ADB282127
ADB271165
ADB282112
ADB255775
ADB265599
ADB282098
ADB232738
ADB243196
ADB257445
ADB267547
ADB277556
ADB239320
ADB253648
ADB282171
ADB233883
ADB257696
ADB232089
ADB240398
ADB261087
ADB249593
ADB264542
ADB282216
ADB261617
ADB269116